Food Research

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INSIDE SCIENCE

The Vital Story of Vitamin B_1

(Thiamine)

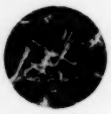
by Science Writer

History. The discovery of vitamin B_1 resulted from research into the cause of beriberi. Almost 50 years passed between Eijkman's discovery of the relationship of the disease to diet and the famous work of Jansen and Donath who first isolated the crystalline vitamin from rice bran.

Within ten years of that first isolation the vitamin's chemical structure was determined and it was successfully synthesized.

Eijkman's work resulted in the development of a theory that beriberi was caused by a lack of some factor in the diet and not by a toxin or infectious agent. This idea was not readily accepted until the growth of dietary knowledge proved it correct.

Isolation and Synthesis. In 1926 Profs. Jansen and Donath accomplished the isolation of crystalline vitamin B_1 from rice bran. In 1931 Windaus and coworkers successfully isolated pure vitamin B_1 and established its empirical formula. In 1936 R. R. Williams, and independently R. Grewe, explained the vitamin's chemical structure. That year, R. R. Williams and J. K. Cline accomplished the synthesis of thiamine which is in wide use today. Andersag and Westphal also synthesized the vitamin in 1936. Another synthesis was described by Bergel and Todd in 1937.



Photomicrograph of B_1 crystals

Chemical and Physical Properties. Thiamine hydrochloride is white, water soluble, with a nut-like, salty taste and yeast-like odor. Its empirical formula is: $C_{12}H_{17}ClN_4OS \cdot HCl$. Thiamine produced by synthesis is identical chemically and in biological activity with that obtained in pure form from nature.

Deficiencies. A deficiency of thiamine is characterized by these symptoms: depression, irritability, fearfulness, lack of initiative and interest, loss of appetite. Symptoms vary since in usual practice deficiencies of other water-soluble vitamins occur. Medical treatment is simple: a sufficient amount of thiamine is administered to relieve symptoms quickly and the physician provides for a continuing adequate intake.

Beriberi victim

A severe deficiency of thiamine leads to beriberi, a serious and sometimes fatal disease. While beriberi is almost a medical curiosity in the United States, it is common in countries in which polished white rice is a staple of the diet.

Human Nutrition Requirements. Thiamine is one of the nutritive elements the human body needs daily and does not store in quantity. The minimum daily requirements established by the U. S. Food and Drug Administration for the prevention of symptoms of thiamine deficiency disease are:

Adults ....1.00 mg.	Children (1-5 incl.)..0.50 mg.
Infants ....0.25 mg.	Children (6-11 incl.).0.75 mg.

The Food and Nutrition Board of the National Research Council recommends the following dietary intake of thiamine for healthy persons in the U. S. A.

Recommended Daily Intake in Milligrams

Age	*Men*	*Women*
25	1.6	1.2
45	1.5	1.1
65	1.3	1.0
Pregnant (3rd trimester)		1.5
Lactating		1.5

The Council recommendations for infants and children vary below and above these figures, based on age and sex. Various illnesses and stress situations can exhaust vital reserves of thiamine. So, for the physician, vitamin B_1 is prepared in various dosage forms and potencies for therapeutic and prophylactic use.

How do human beings receive thiamine? It is widely distributed in foods of animal and vegetable origin, particularly cereal grains and dry legumes. Because of public demand for refined products which millers must meet for obvious economic reasons, a loss of thiamine and other factors occurs during processing. The thiamine loss is overcome through the use of *enrichment* in cereal grain products for which Federal Standards exist, or in other foods such as breakfast cereals, by *fortification* or *restoration*. When enriching, fortifying or restoring, the food processor adds the necessary amount of pure thiamine (and other vitamins and minerals) to the food so that the finished product meets Federal, state and territorial requirements or contributes to the consumer an amount of the vitamin which dietary experts believe significantly useful.

Thiamine is extensively used for the enrichment of cereal grain foods such as white flour, white bread and rolls, macaroni products, farina, corn grits and meal, milled white rice. The story of these uses is delightfully told in a separate brochure which is available on request for reference or educational purposes.

Production. Huge production facilities at the Hoffmann-La Roche plant in Nutley, New Jersey, deliver highest quality thiamine *by the tons*. Roche manufactures thiamine hydrochloride and thiamine mononitrate. These fine products, which equal or exceed U.S.P. specifications, are ideal for use by pharmaceutical makers and food processors. Years of experience in research and manufacture have made Roche the leader in vitamins.

This article is published in the interests of pharmaceutical manufacturers, and of food processors who make their good foods better with essential, health-giving vitamin B_1. Reprints of this and others in the series are available on request. Write the Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 286 St. Paul Street, West; Montreal, Quebec.

SOME ASPECTS OF NUMERICAL SCORING IN SUBJECTIVE EVALUATION OF FOODS[a]

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(Manuscript received January 29, 1955)

There are many different types of objectives in experiments that involve taste-testing *(2, 4, 6, 7, 8)*. A particular type of problem is one in which several variants or test preparations of a food are compared with a standard product. In such experiments the aim is to examine whether the variants differ in some sense appreciably from the standard and also to obtain some objective notion of the extent to which the variants differ amongst themselves. Also, one may wish to know how the variants are ordered with respect to their "distance" from the standard. The "distance" of the variants from the standard can be measured by physical or chemical attributes, but different attributes will lead to different measures of "distance." Because of this, and from general considerations, what frequently is desired is a "distance" measured organoleptically as a composite of all the attributes of the food.

In other cases, a standard may not exist, and one is concerned with an organoleptic rating of the different "varieties" of a food. This problem can arise in what one may term pure consumer acceptance testing and also in food research. In the former case, it is necessary that the judges be a random sample of the population of consumers. In the latter case, one may use what are termed expert judges. In either case, a judge often is asked to score the items being compared on a scale, such as one running from 0 to 5. Alternatively the judge may be asked to record non-numerical evaluations such as *rancid, mealy, yeasty,* and so on.

This study was concerned with a few basic aspects of the procedures of scoring tests. In all the cases to be considered a reference standard was used and the judge was instructed that the identified standard was to be given a particular score, or top score, and that each test preparation was to be given a score, up to and including the top score, which reflected his evaluation of the extent to which the test preparation differed from the standard. The total permitted range of scores was termed the scale and scales of 0-5, 0-10, and 0-100 were used for this purpose. The first question that was examined was whether or not various scales have the same pragmatic value and 3 scales 0-100, 0-10, and 0-5 were evaluated comparatively. Secondly, the effect of order of presentation of substances was examined. Thirdly, the use of different standards was investigated in one respect.

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It is not claimed that a complete answer is given to all the questions which can be raised. The ensuing material provides some evidence on some questions which will, it is hoped, be of interest to users of taste tests involving scoring and those who are concerned with the logical basis of scoring in sensory tests.

METHOD

Reconstituted frozen orange juice was used as the test material. This food was chosen both because of the ease with which a uniform sample could be prepared and because of the ready acceptance of orange juice by the judges. The sweetness of the juice was varied by adding either 16 g. or 40 g. of sugar per quart of reconstituted juice, to give approximately 2% or 5% levels of added sugar. The temperature of the juice at the time of the judging ranged between 2° and 6°C.

The members of the judging panel were senior students in Iowa State College classes in experimental cookery. With the exception of experiments conducted in 1953 wherein latin square designs were used requiring only 12 students out of a class of 14, the entire class (usually 18 students) served as judges in any given series of experiments.

The first series of experiments given in the spring and fall of 1952 was designed to obtain comparative data on the judges' score on the 0-100 and 0-5 scales. In each trial, the judges were given a sample marked control which contained no added sugar and 3 coded samples: one contained no added sugar, one contained 2% added sugar and one contained 5% added sugar. The order of presentation of the samples to the judges was varied in a random manner. In all cases except one, the 0-100 and 0-5 scales were used on alternate days. The judges were instructed to give the highest score on the scale to the sample which most nearly resembled the control or standard sample. They were told to taste the juices in the order presented. The entire test was repeated with 4 different classes. The number of judges in the experiments varied from 16 to 18. There were 6 observations by each judge with each scale and with each level of added sugar. In the first 2 experiments of this series, the judges were given no preliminary training or practice and no information on their day-to-day performance. However, in the last 2 experiments given in 1953, judges had a few practice sessions and were told each day whether or not they had assigned the highest score in the series to the sample which was similar to the standard.

In all the experiments, the judges were not informed as to the composition of the juice and did not know that the same test samples were being submitted for repeated scoring. Also, the judges were not required to record the scores for the first submitted sample before tasting the second, and the second before tasting the third. Intercomparisons were permitted only if the judge tasted the samples again in the order presented, e.g. if, after tasting sample number 3, one wished to compare it to sample 2, then one must taste sample number 1, then sample 2 and finally sample 3.

A second series of experiments given in the spring of 1953 was designed not only to compare the judges' use of 2 scales but also to obtain information on effect of order of presentation. A comparison was made of 6 possible orders of presentation. In this experiment the scales 0-5 and 0-10 were used on alternate days. The arrangement consisted of two 6×6 latin squares with student judges as rows and days as columns for the 0-10 and for the 0-5 scale. The schedule of classes did not permit completion of the sixth judging on the 0-10 scale. At the conclusion of each day's judging, the judges were told whether or not they had assigned the highest score in the series to the sample which was similar to the standard.

A third test was performed to obtain data on the scores for 3 samples, presented at random, 2 of which were the same. This test was a comparison of the following combinations:

Standard	Unknown samples
% sugar	% sugar
None	None, 2, 2
None	None, 5, 5
2	2, 5, 5

The experimental design consisted of two 3×3 latin squares with student judges as rows and days as columns for each of the scales 0-5 and 0-10. As in the second test, the scales 0-5 and 0-10 were used on alternate days. At the conclusion of each day's judging the judges were told whether or not they had assigned the highest score in the series to the sample which was similar to the standard.

ANALYSIS OF THE DATA

Comparison of scales. Exactly how one should compare the scales is not an easy question to answer. We know of no objective criteria which have been used by other workers in the field. The comparison of scales of measurement was discussed by Cochran (3) but essentially only from the point of view of whether or not the scales are related linearly under rather restrictive assumptions. This relation cannot be assumed to hold in the present case.

In the comparison we made, the first step was to consider the relationship between mean values for the test substances. It was presumed that averaging over repetitions of individual judge's scores and experiments would result in a series of numbers which could be regarded as approximately "true scores" on each scale for the preparations. By "true score" here we mean the average score given to the test material with the particular scale and instructions by a population of judges with a large number of evaluations by each judge. The population of judges inherent in the present case is the population of college students who take a particular course in foods. We did not consider any precise statistical tests on the averages because we are aware of no tests which can handle observations which are correlated and subject to differing variances.

The mean scores expressed as percentages of the score for the standard (100, 10, or 5) are given for each experiment in Table 1. First, it is of interest to note that the mean scores have a definite relationship to the percentage of added sugar. This result is similar to results obtained by Hopkins *(5)* with respect to added quinine sulfate and added salt. The relationship of the mean scores to added level of sugar is given for each scale in Figure 1. There do not appear to us to be any advantages which would accrue from the use of any refined statistical procedure such as fitting curves, because

TABLE 1

Mean scores for samples containing varying amounts of sugar

Level of added sugar	Experiment	Mean score expressed as percentage of standard		
		0-100 scale	0-5 scale	0-10 scale
0	Spring '52	94.6	86.0	
	Spring '52	91.4	88.6	
	Fall '52	97.7	96.0	
	Fall '52	96.7	93.8	
	Spring '53	.....	95.8	98.7
	Mean	95.1	92.1	
2%	Spring '52	78.2	62.4	
	Spring '52	78.3	68.6	
	Fall '52	84.7	77.6	
	Fall '52	85.4	71.4	
	Spring '53	.....	78.2	76.0
	Mean	81.7	71.6	
5%	Spring '52	51.7	37.6	
	Spring '52	52.4	35.6	
	Fall '52	62.0	41.6	
	Fall '52	61.1	38.0	
	Spring '53	.....	38.2	43.3
	Mean	56.8	38.0	

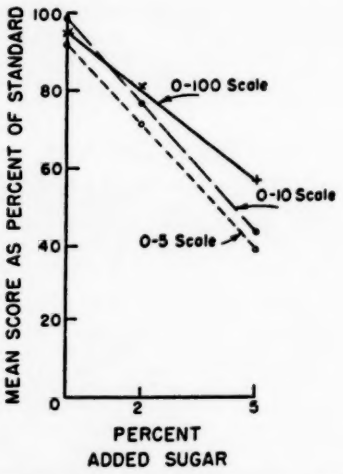

Figure 1. Relationships of scores for the 3 scales to percent added sugar.

the number of levels of added sugar was only 3 and a perfect fit could be obtained by a quadratic expression. The conclusion we draw from this aspect of the evaluation of the data is that even inexperienced judges like those used in the present experiments give results of objective validity when a large number of observations is averaged to reduce the effects of random fluctuations. With experienced judges one would presume that a closer and perhaps sharper relationship would be obtained.

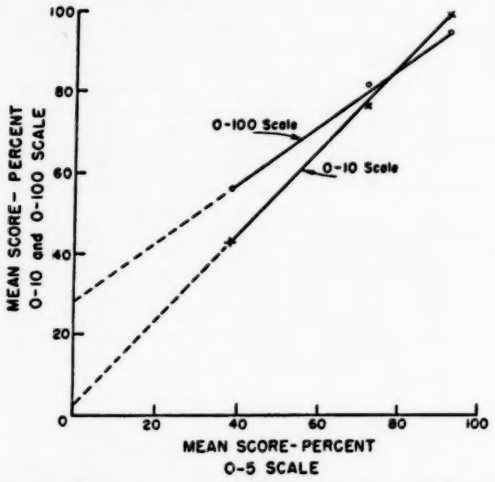

Figure 2. Relationships among mean scores, expressed as per cent of standard, for samples containing 0, 2, and 5% added sugar as determined on the 0-5, 0-10, and 0-100 scales.

Next, we considered the relationships between mean scores on the 3 scales. The results are expressed graphically in Figure 2. There appears to be linear relationships between the true scores on the 3 scales. Roughly it appears that

$$x_{100} = 30 + 14x_5$$
$$x_{10} = 0.4 + 2.04x_5$$

The 0-10 scale and the 0-5 scale gave results which are almost related by a constant multiplier; in fact, it was almost the case that the score on the 0-10 scale was twice the score on the 0-5 scale. In the case of the 0-100 scale it appears that a large proportion of the lower half of the scale was not used by the judges, roughly the bottom 40 points of the scale were not used. As a result a difference of 1 unit on the 0-5 scale does not correspond, with this population of judges, to a difference of 20 units on the 0-100 scale.

The question which then arises is whether or not one scale is preferable to another on the basis of reproducibility and sensitivity. The scores of a judge with a particular scale may be perfectly reproducible in the sense of there being no variation from day to day but may have no sensitivity in that the same score is given to all test preparations. Suppose the scores x_1, x_2 on two scales denoted by '1' and '2' were related so that

$$x_1 = X_1 + e_1,\ V(e_1) = \sigma_1^2,$$
$$x_2 = X_2 + e_2,\ V(e_2) = \sigma_2^2,$$

where x_1, is the observed score, X_1 is the true score, conceptually defined as the average of an indefinitely large number of independent observed scores for a particular test preparation with a particular scale and scoring instructions. Also suppose that $X_1 = aX_2$, then the direct measure on Scale '1' would have a standard deviation of σ_1 and the measure from scale '2' calibrated to scale '1' would have a standard deviation equal to $a\sigma_2$. Scale 1 would be the more accurate if σ_1 is less than $a\sigma_2$. This can be put alternatively in the following form: if the coefficient of variation (CV) for scale 1 is less than the coefficient of variation for scale 2, then scale 1 is more accurate. This follows because

$$CV_1 = \frac{\sigma_1}{X_1}$$

$$CV_2 = \frac{\sigma_2}{X_2} = \frac{a\sigma_2}{X_1}$$

so if CV_1 is less than CV_2, $a\sigma_2$ is less than σ_1.

For the comparison of the 0-10 and 0-5 scales it, therefore, seems appropriate to consider the average coefficient of variation, where the coefficient of variation is estimated for each judge and then averaged over judges and over experiments. The assumption is made here that there are no trends in the scores. The results are given in Table 2. The coefficient of variation for the 0-100 scale is given for what it may be worth.

TABLE 2

Average coefficients of variation for the three scales

Level of added sugar	Scales		
	0-100	0-10	0-5
0	6.5	2.6	14.1
2%	14.5	18.8	23.3
5%	27.5	42.7	51.2

Strictly speaking, a comparison is useful only between the 0-5 and 0-10 scales, and it appears that the 0-10 scale gives a more accurate result.

For the comparison of the 0-100 scale and 0-5 scale the situation is that

$$x_{100} = 30 + 14x_5$$

In this case a standard deviation of say σ_5 on the 0-5 scale, therefore, would be equivalent to a standard deviation of $14\sigma_5$ on the 0-100 scale. Our procedure is, therefore, to get the mean squares between repetitions of the scores of each judge, average these over all judges and take the square roots to give average standard deviations on each scale for each test preparation. (Actually the mean squares varied considerably and a more precise evaluation of the situation, which the present data do not justify, might require a different methodology). The estimated average standard deviations are given in Table 3.

TABLE 3

Average standard deviations within judges for 0-100 and 0-5 scales

Level of added sugar	0-100 scale	0-5 scale
0	9.0	0.78
2%	12.7	0.89
5%	13.1	0.90

The present reasoning suggests that if the standard deviation for the 0-100 scale is less than 14 times the standard deviation for the 0-5 scale then the 0-100 scale is the more accurate and vice versa. Therefore, it appears that on the grounds of accuracy the two scales are essentially the same. Whether this result would be found in more elaborate tests with experienced judges is a matter of conjecture.

Another method of comparing the scales arises from the point that the main purpose of the scoring test as used here is the detection of differences. If there were only two levels of added sugar, one would evaluate for each judge the criterion (mean difference/standard deviation of difference) and the scale with the larger value would be the more sensitive. The natural generalization is to consider the analysis of variance for the scores of a judge:

	Mean expected square	Mean square
Between days	D	
Between levels of added sugar	T	$\sigma^2 + r\sigma_1^2$
Residual	E	σ^2

We then estimate σ_1^2/σ^2 by $C = (T/E - 1)/r$. If the number of repetitions were the same throughout, one would merely have to calculate F equal to T/E. The scale which gives the higher value of the criterion would be the more sensitive for the particular judge. The quantity C was calculated for each judge in each series of tests and the values of C for the 2 scales under comparison in a test were compared. The results are

TABLE 4

Comparison of sensitivities of scales

	Number of judges for which scale is more sensitive		
	0-5	0-10	0-100
Spring 1952	3		13
Spring 1952	7		5
Fall 1952	2		16
Fall 1952	10		8
Spring 1953	5	9	
Spring 1953	7	7	

given in Table 4. It is curious that there is not a consistent difference between the 0-5 and 0-100 scale. For 2 sets of judges the 0-100 scale is clearly superior to the 0-5 scale while for the other 2 sets no clear-cut difference can be claimed. The origin is presumably the difference in instructions.

Effect of order of presentation of samples. The experiments in which the samples were judged in various orders in the latin square design were analyzed according to the analyses of variance shown in Table 5. In the experiments with the 0-5 and 0-10

TABLE 5

Analysis of variance for latin square design to test effects of order of presentation

Source of variation	Degrees of freedom	
	0-10 scale	0-5 scale
Squares	1	1
Days in squares	8	10
Judges in squares	10	10
Order within squares	10	10
Error within squares	30	40
Total	59	71

scale, the analysis used was that for an incomplete latin square. Analyses were not calculated for the scores for samples without added sugar since in only 5 out of 60 judgings on 0-10 scale and 9 out of 72 judgings on 0-5 scale were the scores less than 10 or 5, respectively. The test of significance used was the ratio of the mean square for orders within squares to the error within squares. The order of presentation was found to affect the scores given to the samples containing 2% added sugar and 5% added sugar, the significance levels being 1% and 5% in one experiment. The adjusted treatment means for the 2% and 5% levels of sugar on the 0-5 and the 0-10 scales are shown in Table 6. The main feature of the results is that the 2% samples received

TABLE 6

Means for 2% and 5% added sugar for each order of presentation on 0-10 and 0-5 scales

Order of presentation	Treatment means[1]			
	2% added sugar		5% added sugar	
	0-10 scale	0-5 scale	0-10 scale	0-5 scale
A 0-5-2	7.93	4.25	4.12	1.58
C 5-2-0	7.91	4.25	3.81	1.92
E 2-5-0	7.75	4.08	3.75	1.84
B 2-0-5	7.33	3.92	3.23	1.42
F 0-2-5	6.85	3.75	3.81	2.08
D 5-0-2	6.83	2.75	3.89	1.42

[1] Standard error of treatment difference (in means) for:
2% added sugar on 0-10 scale = 0.68
2% added sugar on 0-5 scale = 0.34
5% added sugar on 0-10 scale = 0.84
5% added sugar on 0-5 scale = 0.30.

higher scores when they followed the 5% samples than when they followed 0% samples. On the other hand, the 5% samples, as scored on the 0-5 scale, received higher scores when they followed the 2% samples than when they followed the 0% samples. This result lends support to the idea frequently expressed that a tester can compare only 2 samples in a test. However, problems remain because there is then the difficulty of deciding if there is a "true score" for a sample and this difficulty does not appear to have been resolved in the literature.

Choice of standard. Another question which is important in scoring tests is whether the score associated with a sample C (in this case the 5% level of sugar) when it is scored directly against sample A (in this case no added sugar) is the same as the score for sample C that is reached indirectly by scoring an intermediate level against A and then scoring C against that intermediate level. Accordingly, the data from the 3×3 latin squares were analyzed and the mean differences for 0% vs. 2%, 0% vs. 5%, and 2% vs. 5% were calculated. A "t" test was used to determine whether the difference for 0% vs. 2% plus the difference for 2% vs. 5% differed from the difference for 0% vs. 5%. The resulting values of "t" were found to be nonsignificant at the 5% level which indicated that the judges' evaluation of the highest level of sugar was the same whether it was evaluated stepwise or directly against the standard containing no sugar.

DISCUSSION

These experiments have been considered from the standpoint of the examination of criteria for choice of scale and for choice of a particular experimental design. Three criteria were used. The first was the average coefficient of variation within the judges. It was seen that this criterion can be used to compare the 0-5 and 0-10 scales, with the 0-10 scale having the lower value. The second criterion was the average standard deviation within judges weighed against the relationship between scales. This was applied to the 0-5 and 0-100 scales and gave no basis for preferring one of these to the other. The third criterion was the sensitivity of the judges to the differences imposed in the experiments. On this basis there was little difference between the 0-5 and 0-10 scales, but between the 0-5 and 0-100 scales, although there was little difference in 2 of the 4 experiments, there was a clear superiority of the 0-100 scale in the other 2 experiments. This ambiguity in the comparison of the 0-5 and 0-100 scales is unresolved.

The relationship between the order in which samples are evaluated and the scores that are assigned is another consideration in the design of scoring tests. Boggs *(2,* p. 86) pointed out that in tasting samples containing sulfite the acuity of the taster decreased after tasting the first sulfite sample. However, Bliss, Greenwood, and McKendrick *(1)* reported that an analysis of covariance indicated that the order of presentation did not affect the judges' rating of potatoes for mealiness.

The effect of order of presentation was clearly established in the studies reported herein. The differences observed are reasonable in that a sample in which there is only a small deviation from the standard is given a higher score when compared to the previous sample which deviates to a greater degree from the standard than in the alternative case. Also the score attached to a sample following a non-standard sample is higher than when it follows a standard sample.

The third set of experiments gave no indication that the score for 5 vs. 0 was different from the sum of the scores for 2 vs. 0 and 5 vs. 2, suggesting that scores may be obtained in a stepwise fashion.

SUMMARY

The aim of the investigation described was to obtain some basic information on the effectiveness of different scales of numerical scoring in subjective evaluation of foods. With a view to obtaining extremes which might shed light on the matter, scales of 0-5, 0-10, and 0-100 were used on reconstituted orange juice with 3 levels of added sugar. It was found that essentially the mean score over a group of judges was related linearly between scales, so that the population of judges could be assumed to be measuring the same attribute or combination of attributes on the different scales. Therefore, the scales were compared according to: a, coefficient of variation; b, standard deviation within judges; and c, sensitivity. The general coclusion is that the 0-100 scale is at least as good as the 0-5 scale and in some cases was better. The 0-10 scale was better than the 0-5 scale. The 0-10 and the 0-100 scales were not compared with the same group of judges so that no choice between these is indicated.

A further aim was to study the effects of order of presentation of samples. The 6 possible orders of presentation of the 3 levels of added sugar were compared in latin squares. It was found that the mean score given to the intermediate level of added sugar samples was affected by the order of presentation.

A third aspect examined was the extent to which comparisons between a number of samples can be made in pairs for 3 samples, A, B & C, with the 3 possible paired tests of A & B, A & C, and B & C. The results indicated that the difference for A vs. C was essentially equal to the sum of the differences of A vs. B and B vs. C. On this evidence, then, scoring by paired tests gives consistent results.

Acknowledgments

The technical assistance of Mrs. Helen Gill and Mrs. Dorothy Rossing is gratefully acknowledged.

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HEAT PROCESSING OF BEEF. PART VIII. STERILIZING EFFECTS OF HIGH TEMPERATURE PROCESSES[a]

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The ultimate purpose of an investigation of physical properties and attempts to determine laws governing the processing of foodstuffs is to make available results which may be used in the improvement of a process leading to a safe and palatable product. In this series of papers on heat processing of beef *(5, 6, 7, 8, 9, 10, 12)*, Hurwicz and Tischer attempted to investigate the laws governing the thermal process and to determine some physical characteristics of beef. Objective measurements of quality were discussed in Part III *(12)*, with only a brief consideration of the sterilizing effects of the process. Since these effects are of paramount importance, this investigation was undertaken to determine the sterilizing effects of high temperature processes. It is the purpose of the investigation to present and to evaluate data on the distribution of F_o values in 300 × 308 cans packed with beef and processed in steam; and furthermore to evaluate the contribution of residual heating *(10)* to the sterilizing effect.

Evaluation of the sterilizing effect of heat processing for the conditions prevailing during this experiment assumes two important aspects: (a) It furnishes information on the effects of high processing temperatures for short times on the sterilizing value of the process and (b) it makes available some information on the distribution of F_o values within a container during heat treatment to permit the evaluation of the sterilizing effect for the whole contents of the can. In both cases the information in the literature is either scarce or not available.

EXPERIMENTAL PROCEDURE

The experimental procedure used in processing of round of beef and the design of the experiment were described in detail in Parts III and IV *(12, 7)* of this series and its description would be repetitious, as data presented here were gathered during the same experiment.

The sterilizing values (F_o) were determined graphically and also calculated by the "General Method" *(2)*. The F_o values were determined for the 6 temperatures investigated and for all of the processing times used; however, only 4 out of the 12 times within each temperature were presented in Table 1 for all 25 locations tested in the container. The locations are given in the table in terms of their r and z cylindrical coordinates. Because of the variation observed, the results are given mostly in tabular and graphical form with the interpretation restricted to observation of trends or in some cases to approximate visual evaluation of functional relationships. No attempt was made to estimate parameters.

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TABLE 1

Distribution of sterilizing values (F_o) for four processing times (t_1) and six processing temperatures (RT) (3 cuts average)

t_1 (min.)	r[a] (cm.)	z[b] (cm.)						
		2.75	2.00	1.00	0.00	−1.00	−2.00	−2.75
		Mean F_o values						
		RT = 225°F.						
50	0	.....	0.181	0.095	0.111	.....	0.150	0.281
	1	0.335	0.164	.....	.....	0.125	.....	0.274
	2	.....	0.171	0.195	0.128	.....	0.241	0.211
	3	.....	.....	0.299	0.305	0.339	.....	.....
70	0	1.712	.....	.....	0.841	0.825	.....	.....
	1	1.208	0.664	.....	.....	.....	1.116	1.020
	2	.....	.....	0.873	0.601	0.956	0.913	1.096
	3	.....	.....	0.904	1.256	0.991	.....	.....
100	0	2.639	1.865	.....	9.183	2.263	2.191	.....
	1	.....	2.482	.....	1.763	.....	2.306	.....
	2	.....	1.921	.....	.....	.....	.....	0.221
	3	.....	.....	.....	.....	2.216	2.808	.....
120	0	1.369	3.614	.....	2.386	1.507	3.799	.....
	1	.....	3.008	1.505	3.380	2.853	1.585	.....
	2	.....	3.872	.....	2.763	.....	.....	3.148
	3	.....	1.632	3.198	.....	.....	1.744	.....
		RT = 243°F.						
42	0	.....	.....	0.469	0.439	.....	.....	.....
	1	0.919	1.125	.....	0.562	0.538	.....	1.144
	2	.....	1.044	.....	.....	0.531	0.919	1.594
	3	.....	.....	2.838	1.200	3.544	.....	.....
67	0	13.006	.....	.....	7.891	7.550	.....	.....
	1	10.171	.....	8.014	.....	3.725	.....	9.019
	2	.....	.....	8.438	11.288	7.762	8.721	.....
	3	.....	10.906	.....	9.8	5.775	.....	.....
84	0	15.794	14.444	14.356	15.190	11.075	16.588	18.238
	1	.....	19.916	.....	12.981	.....	11.784	.....
	2	.....	16.625	.....	.....	.....	.....	15.850
	3	.....	10.300	.....	.....	.....	18.975	.....
		RT = 225°F.						
101	0	12.019	.....	16.856	16.139	.....	.....	21.931
	1	.....	.....	9.125	15.954	18.125	.....	.....
	2	.....	.....	.....	18.234	.....	.....	20.438
	3	.....	15.638	23.006	.....	22.781	15.931	.....
		RT = 261°F.						
34	0	.....	.....	1.182	0.831	.....	2.125	10.062
	1	6.250	2.188	.....	.....	1.168	.....	11.688
	2	.....	2.125	9.250	0.750	3.750	1.625	.....
	3	.....	.....	4.812	4.188	2.876	.....	.....
54	0	48.436	.....	.....	33.688	22.062	.....	.....
	1	19.322	44.438	22.212	.....	.....	31.412	18.500
	2	.....	.....	11.812	24.608	11.312	12.286	21.938
	3	.....	62.938	.....	21.125	52.438	.....	.....

[a] r = distance from vertical axis
[b] z = distance from central horizontal cross-section

TABLE 1 (continued)

$t_{\frac{1}{2}}$ (min.)	r[a] (cm.)	z[b] (cm.)						
		2.75	2.00	1.00	0.00	−1.00	−2.00	−2.75
				Mean F_o values				
				RT = 279°F.				
68	0	97.562	81.312	108.438	71.521	.....	.....	121.250
	1	.....	.....	.....	90.125	81.062	52.750	.....
	2	.....	107.875	.....	.....	.....	.....	.....
	3	.....	125.875	.....	.....	.....	114.500	.....
82	0	121.062	118.562	79.625	132.456	.....	83.675	113.538
	1	.....	166.212	.....	.....	.....	.....	.....
	2	.....	36.625	.....	142.662	.....	.....	197.875
	3	.....	169.325	179.312	.....	172.436	132.312	.....
				RT = 279°F.				
31	0	.....	14.75	9.50	6.25	.....	25.00	142.75
	1	17.00	7.75	.....	.....	2.75	.....	9.25
	2	.....	31.00	.....	4.25	3.15	3.50	17.50
	3	.....	.....	19.25	158.50	40.50	.....	.....
42	0	280.00	.....	.....	214.00	29.75	.....	.....
	1	514.50	85.25	51.70	.....	.....	.....	.....
	2	.....	.....	83.150	369.75	102.50	117.75	156.25
	3	.....	136.75	248.65	174.25	341.00	149.75	.....
52	0	785.00	201.75	234.00	330.67	311.00	315.50	371.50
	1	.....	534.85	332.75	165.25	170.75	.....	.....
	2	.....	258.00	.....	129.10	.....	.....	378.50
	3	.....	.....	.....	.....	352.00	608.25	.....
62	0	619.25	35.50	.....	379.083	277.00	.....	.....
	1	.....	.....	376.65	.....	360.60	.....	.....
	2	.....	568.50	.....	435.50	.....	.....	681.00
	3	.....	972.65	.....	.....	809.00	995.60	.....
				RT = 297°F.				
21	0	.....	.....	0.41	0.36	.....	.....	.....
	1	25.0	5.1	.....	.....	0.33	.....	2.9
	2	.....	9.9	.....	.....	.....	1.4	16.9
	3	.....	.....	33.5	27.5	22.9	.....	.....
29	0	.....	.....	.....	13.0	1.8	.....	.....
	1	145.0	560.0	3576.5	.....	.....	.....	47.6
	2	.....	.....	21.0	15.0	21.1	41.4	.....
	3	.....	276.5	1498.5	617.5	563.6	101.3	.....
36	0	3771.5	2855.0	347.5	1439.6	.....	560.0	.....
	1	.....	.....	.....	233.0	146.5	75.0	.....
	2	.....	.....	.....	116.5	.....	.....	900.0
	3	.....	123.50	296.5	.....	.....	880.0	.....
43	0	672.5	.....	.....	142.0	345.0	346.0	1210.0
	1	.....	.....	233.5	.....	739.5	806.0	.....
	2	.....	516.5	.....	558.5	.....	.....	.....
	3	.....	915.0	2010.0	.....	2660.0	2318.5	.....

[a] r = distance from vertical axis
[b] z = distance from central horizontal cross-section

TABLE 1 (concluded)

t_1 (min.)	r^a (cm.)	z^b (cm.)						
		2.75	2.00	1.00	0.00	−1.00	−2.00	−2.75
		Mean F_o values RT = 315°F.						
12	0	.....	.....	.....	0.036	.....	0.201	0.058
	1	0.016	.....	.....	.....	0.034	.....	0.101
	2	.....	0.812	.....	0.031	.....	.....	15.83
	3	.....	.....	17.79	.....	1.82	.....	.....
16	0	365.0	.....	.....	0.16	.....	.....	.....
	1	4587.5	.....	.....	.....	.....	.01	.....
	2	.....	.....	0.17	225.0	0.33	0.71	.....
	3	.....	20.7	493.2	7.1	1.3	11.4	.....
20	0	5432.5	145.9	4.1	32.0	179.8	37.4	.....
	1	.....	1134.2	.....	2.4	12.7	9.2	.....
	2	12.7	.....	.....	.....	.....	.....	150.7
	3	.....	421.7	354.2	.....	75.7	88.4	.....
24	0	5142.5	.....	9.1	43.5	.....	35.2	648.7
	1	.....	3682.5	7.7	5.3	9.6	5267.5	.....
	2	.....	.....	.....	.....	.....	.....	13782.5
	3	.....	4443.2	.....	.....	259.2	1895.7	.....

The authors believe that the scarcity of data on F_o distributions in canned meats (or any other canned products) for the whole container will make the presentation of the experimental results, even though in tabular and graphical form only, of value to the research worker or to the canner working on the determination of a satisfactory canning process, especially on the volume-lethality basis *(3, 4, 5, 11)*.

RESULTS AND DISCUSSION

F_o distribution. F_o values for the 6 temperatures used and for 4 of the 12 times within each temperature are given in Table 1 for all 25 locations investigated.

The mean value of F_o for the 3 cuts of the round of beef (Table 1) indicate, in spite of the large variations observed, that the lowest sterilizing effect does not necessarily occur at the center of the container. It seems to be displaced off the central vertical axis and above or below the central horizontal axis to form an area of low F_o values on the vertical cross-section of the can, similar to the greatest heating lag area observed in temperature distributions *(8)*. This effect persists through all temperatures and processing times. The low F_o value area upon 360° revolution of the cross-section around the central verical axis forms a doughnut-shaped volume of low sterilizing values. This phenomenon may be explained as a secondary effect of the occurrence of theoretically unexpected temperature distributions [proven previously to be real and not chance effects *(8)*] or as an effect of the anisotropic structure of meat, or chemical and moisture changes occurring in the medium due to the processing operation, and also as a result of the searing at the boundaries of the piece of meat sealed in the can. It may be noted that this phenomenon is especially pronounced for higher processing temperatures, which leads to another explanation of the low F_o value area. The outside region of the can undergoes, during the heating phase of the process, a more severe heat treatment but during the cooling phase cools off more rapidly than other parts of the can volume and the lag in cooling is less pronounced. On the other hand, even though the central part of the can volume does not heat up rapidly during the heating phase of the process it cools slower than the outside; in addition, during the period of time just after the cooling water is started, the central portion of the contents of the can instead of cooling down actually continues to heat *(10)*. Thus the contribution of the cooling phase to the F_o value although small for the outside region becomes very important for the central portion. The remaining portion of the contents between

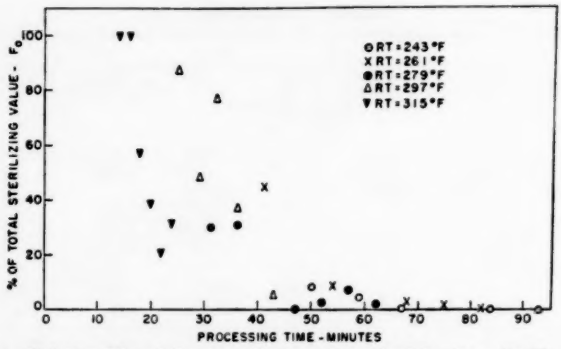

Figure 1. Relationship of the contribution of residual heating (to F_o values) and processing time.

central and outside regions displays characteristics intermediate to those described for the other two zones. These phenomena are more pronounced at high temperatures. The evidence in this experiment indicates that such behavior may result in a volume of low F_o values not at the center of the can.

Contribution of residual heating during cooling phase to F_o values. The importance of the cooling phase is illustrated in Figures 1 and 2 and Table 2. The percentage of sterilizing value due to residual heating does not include the total contribution of the cooling phase to the sterilizing value. It includes only the effect which is caused by continued heating, called henceforth residual heating *(10)*, at a given location in spite of the cut-off of the steam; it does not include the portion of the F_o value which would be contributed to the total sterilizing effect if cooling started instantaneously after the cut-off of the steam. In other words the total sterilizing effect may be divided into 3 parts: the first occurs during the heating period, the second part is contributed because of the displacement of the cooling curve resulting from residual heating after the cooling phase has been started, and the third part is contributed during the cooling period on the assumption of instantaneous cooling. The contribution of the second part of the thermal process is most pronounced at the locations of greatest heating lag and is practically zero in the outside region of the can. Residual heating effects have been evaluated for selected points representing the three arbitrarily chosen regions in the can (central, intermediate, and outside region) for times yielding sterilizing values above $F_o = 1$. These effects were evaluated for 5 temperatures only, since inspection of data readily showed them to be negligible for the processing temperature of 225°F. Table 2 also gives additional evidence that the area of low F_o values was a true effect

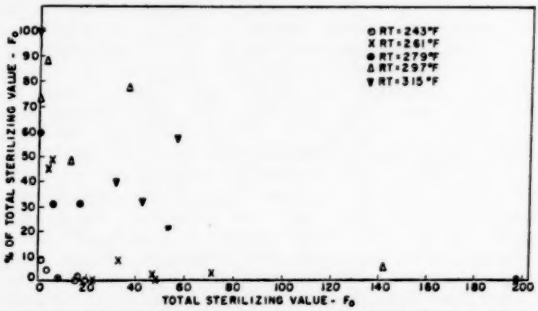

Figure 2. Relationship of the contribution of residual heating (to F_o values) and sterilizing effect.

as it coincided with the area where the contribution of the residual heating to the sterilizing effect (F_o) was the highest.

From Figure 1 and Table 2 it may be observed that the highest percentage contribution occurs for each processing temperature for short processing times and decreases rapidly for longer processing times. The rate of decrease increases with the increasing temperatures and also the contribution is higher for higher temperatures. Figure 1 does not offer the best means of comparison among various temperatures because of different F_o values of the processes. The plot of the percentage of F_o contributed by the residual heating effect of the process (Figure 2) indicates that this contribution decreases with the increase in total F_o value of the process for all temperatures and that for the same F_o value of the process the contribution increases rapidly in the temperature range from 225-298°F. and then either remains unchanged or even slightly decreases for the processing temperature of 315°F. This effect is especially pronounced for locations (11 and 12) Figure 3 which exhibited the greatest heating lag although

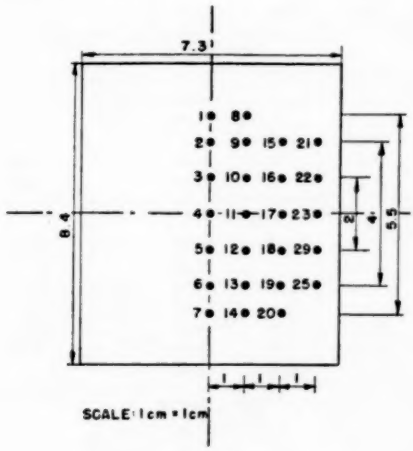

Figure 3.

it may not be as readily seen on Figure 2 for the center point (which was presented in the text because of the larger number of determinations made and involving less interpolations). The maximum contribution at locations 11 and 12 occurred at 297°F.

These facts bear out the necessity for evaluation of the sterilizing effects for the whole volume of the can and not only at the center point; they also demonstrate an underestimation of sterilizing effect of Ball's "Formula Method" *(1)* used for process determination and evaluation, where instantaneous cooling is assumed. This underestimate may range from 15 to 50% for 261°F. retort temperature to 55 to 90% for 297°F. and 315°F. retort temperatures when the F_o range considered is from 1 to 30. This F_o range would include, for practical purposes, all the useful canning processes.

The discussion of the residual heating effects may also explain the relatively low gradient of F_o values from the outside to the center which may be observed from Table 1 of the F_o distribution. On the average, the highest F_o value occurring in the outside region did not exceed the lowest F_o value at any place in the can by more than a factor of three (for the same process) with the notable exception of the retort temperatures of 297°F. and 315°F. where the gradient was considerably higher.

Relationship of sterilizing effects, processing times and temperatures. To emphasize further the variation in determined F_o values, Table 3 was compiled showing, for each of the 6 processing temperatures, the average F_o values for 9 or 10 processing times for 8 selected locations in the can. It would be expected that the F_o values

TABLE 2

Percentage of total sterilizing value, F_o, of a thermal process contributed by residual heating (during cooling phase) for selected locations in the can

Processing time (min.)	Location							
	4	11	12	17	19	6	24	14
	Percent of F_o during cooling							
	%	%	%	%	%	%	%	%
	RT = 243°F.							
50	8.73	(29.21)[a]	25.96	.....	0.00	.....	.....	.....
59	4.45	.....	.....	26.47	.....	.....	0.00	22.19
67	0.55	.....	30.09	11.99	0.00	.....	26.34	0.00
84	0.00	12.33	.....	.....	.....	9.02	.....	.....
101	1.81	0.00	0.00	0.00	.....	.....	0.00	.....
	RT = 261°F.							
41	43.25	51.40	43.37	.....	0.00	.....	.....	.....
48	0.00	.....	.....	0.00	24.35	.....	0.00	.....
54	3.54	.....	.....	5.72	0.00	.....	3.98	0.60
68	2.78	0.00	0.00	.....	.....	.....	.....	.....
75	2.29	3.47	0.00	0.00	.....	2.62	0.00	.....
82	0.00	.....	.....	0.00	.....	6.26	0.00	.....
	RT = 279°F.							
31	30.40	.....	96.35	50.59	0.00	35.40	0.00	32.43
36	31.13	.....	.....	20.86	.....	.....	0.00	28.04
42	0.00	.....	.....	0.00	36.94	.....	0.00	.....
47	0.00	.....	.....	0.00	0.00	.....	0.00	0.00
52	3.29	19.27	0.00	0.00	.....	12.56	0.00	.....
57	7.28	5.00	0.00	0.00	.....	4.18	0.00	.....
62	2.05	.....	0.00	0.00	.....	.....	0.00	.....
	RT = 297°F.							
25	88.31	.....	.....	92.46	92.32	.....	.....	.....
29	48.57	.....	.....	0.00	73.99	.....	0.00	73.95
32	76.07	.....	.....	51.74	84.75	.....	.....	68.90
36	37.19	69.31	0.00	0.00	.....	50.89	.....	.....
43	5.69	.....	0.00	0.00	.....	22.03	0.00	.....
	RT = 315°F.							
14	(100.00)	.....	.....	(100.00)	98.34	.....	29.31	.....
16	(100.00)	.....	.....	99.74	(95.35)	.....	100.00	86.63
18	57.26	.....	0.00	0.00	99.32	.....	0.00	97.74
20	39.82	100.00	72.92	.....	.....	79.80	0.00	.....
22	21.26	58.22	64.44	35.65	.....	67.64	0.00	.....
24	32.06	95.26	65.49	.....	.....	78.72	0.00	.....

[a] $F_o < 1$

would increase monotonically with the increase of processing time. The rate of increase would be more rapid as the processing times become longer. This was the case when F_o values were plotted for each temperature separately as a function of processing time and when a curve was fitted to the experimental data for the center point (Figure 4). However, in the temperature range of 225-279°F. a decrease in the rate of change was observed for long processes. Inspection of the data contained in Table 3 and of the visually fitted curves in Figure 4 shows, however, that in many cases the F_o values (averaged over 3 determinations) are much larger for shorter processing times than for the longer ones, which is definitely against expectation and demonstrates the varia-

TABLE 3

Sterilizing values (F_o) of thermal processes for selected locations in the can

Processing time (min.)	Location							
	1	2	4	5	9	17	20	24
	Sterilizing value (F_o)							
				RT = 225°F.				
40	.....	0.05	0.04	0.08	0.07	0.05	0.12	0.20
50	.....	0.18	0.11	0.34	0.16	0.13	0.21	0.34
60	.....	0.55	.....	1.07	0.51	.....	.....	0.52
70	.....	.....	0.84	1.21	0.66	0.60	1.10	0.99
80	.....	.....	0.95	1.16	.....	2.32	0.13	1.52
90	2.14	.....	1.53	1.68	1.69	1.65	2.17	2.17
100	2.64	1.86	1.98	.....	2.48	.....	0.22	2.22
110	3.58	2.07	2.32	.....	3.42	2.44	2.57	3.05
120	1.57	3.61	2.39	.....	3.01	2.76	3.15	.....
	3	**4**	**7**	**9**	**11**	**20**	**21**	**25**
				RT = 243°F.				
25	.....	.....	1.20	.....	.....	.....	.....	0.12
34	0.09	0.18	.....	0.30	0.06	0.27	.....	.....
42	0.47	0.44	.....	1.13	0.56	1.59	.....	.....
50	1.52	1.10	2.26	1.22	1.21	2.73	.....	.....
59	.....	3.46	.....	.....	.....	4.13	9.54	.....
67	.....	7.89	.....	.....	.....	.....	10.91	.....
76	.....	7.65	.....	10.57	.....	11.97	14.12	15.56
84	14.36	15.19	18.24	19.92	12.98	15.85	10.30	18.98
93	18.44	19.68	27.96	18.88	18.34	15.23	.....	.....
101	16.86	16.14	21.93	.....	15.95	20.44	15.64	15.93
	1	**3**	**4**	**8**	**12**	**16**	**19**	**23**
				RT = 261°F.				
21	0.50	.....	4.91	.....	.....	.....	.....	0.56
27	.....	.....	0.25	0.94	.....	0.79	0.25	1.19
34	.....	1.19	0.83	6.25	1.19	9.25	1.63	4.19
41	.....	4.50	4.19	19.56	5.19	9.69	14.50	22.04
48	.....	.....	21.75	25.19	.....	30.63	16.94	38.00
54	48.44	.....	33.67	19.31	.....	11.81	12.29	21.13
61	123.94	.....	48.95	74.46	51.38	46.75	41.38	54.94
68	97.56	108.44	71.52	.....	81.06	.....	.....	.....
75	7.81	86.83	47.20	.....	45.63	.....	.....	.....
82	121.06	79.63	132.46	.....	.....	.....	.....	.....
	2	**4**	**8**	**10**	**17**	**20**	**22**	**24**
				RT = 279°F.				
21	.....	.....	11.50	.....	.....	4.75	.....	6.50
26	10.00	8.63	9.00	.....	.....	13.50	6.50	10.00
31	14.75	6.25	17.00	.....	4.25	17.50	19.25	40.50
36	.....	16.63	56.50	3.75	64.00	.....	438.50	584.25
42	.....	214.00	514.50	51.70	369.75	156.25	248.65	341.00
47	.....	198.67	404.75	56.25	144.75	291.00	460.25	533.85
52	201.75	330.67	.....	332.75	129.00	378.00	.....	352.00
57	355.00	295.35	.....	.....	73.50	948.00	.....	1126.00
62	35.50	379.08	.....	376.65	435.50	681.00	.....	809.00

TABLE 3 (continued)

Processing time (min.)	Location							
	1	4	5	9	10	17	21	22
	Sterilizing value (F_o)							
	RT = 297°F.							
11	0.45	.....	0.54	.....	.....	.....	0.91	.....
14	.....	.....	.....	.....	.....	.....	.....	4.45
18	.....	0.65	.....	.....	.....	.....	.....	9.94
21	.....	0.36	.....	5.10	.....	.....	.....	38.50
25	.....	2.68	571.50	33.15	.....	2.45	.....	451.50
29	.....	13.03	1.84	560.00	3576.50	15.00	276.50	1498.50
32	230.00	36.75	43.50	125.00	45.00	2058.50	.....	565.00
36	3771.50	1459.50	.....	.....	.....	118.50	123.50	896.50
40	.....	416.25	528.50	.....	295.00	636.50	1880.00	1630.00
43	678.50	142.00	.....	.....	233.50	558.50	915.00	2010.00
	1	4	8	9	12	20	22	24
	RT = 315°F.							
8	.....	.....	1.49	.....	.....	0.09	6.84	0.32
10	.....	.....	0.15	0.02	.....	0.03	1.42	33.93
12	.....	0.04	0.02	.....	0.03	15.83	17.79	1.82
14	.....	0.15	21.88	.....	.....	3.89	82.43	4.98
16	365.00	0.16	4387.50	4992.50	.....	.....	488.25	1.26
18	.....	56.90	1466.70	2.10	16.24	.....	188.58	41.50
20	5432.50	31.96	.....	1134.25	12.67	150.68	354.25	75.75
22	781.75	52.93	.....	5507.50	9.00	83.18	1115.00	380.00
24	5142.50	43.52	.....	3682.50	9.59	13782.50	.....	259.25

bility of results. It may be noted, however, from inspection of the visually fitted curves in Figure 4 where log F_o was plotted against processing time for 6 temperatures, that the log F_o may be expressed by two linear functions for each of the temperatures, of the general form of

$$\log F_o = (a_1 + b_1 t) + [a_2 + b_2 (t - t_1)]$$

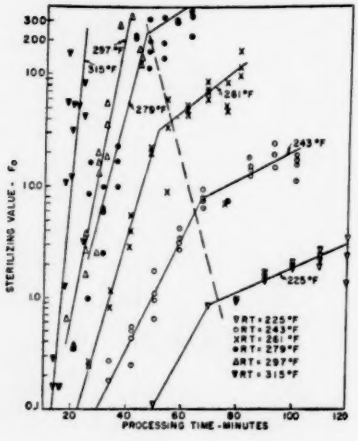

Figure 4. Relation of sterilizing values and processing times for 6 retort temperatures.

where t_i is the processing time when the function changes slope. For the shorter processing times the slope (b_1) of the straight line is larger than the slope (b_2) for longer processing times. Also the points of change of slope for the 6 temperatures may be related by a linear function.

In view of the variation encountered no attempt was made to relate the F_o values, processing times and processing temperature by a specific mathematical relationship. In spite of the variations observed it seems doubtful that the various phenomena discussed above were only chance events since the trends persisted through all of the processing conditions. The reader is nevertheless cautioned to remember that a single determination of an F_o value is useless and that a small arbitrary number of F_o values gives no guarantee that the lowest F_o value in the sample will actually be the safest. On the basis of the data collected in this experiment the authors are not prepared, nor is it within the scope of this work, to make recommendations as to the size of the sample necessary to obtain precise information. An investigation of this problem seems to be very urgent and statistical methods are available to furnish the answer to the question.

By means of Figure 4, drawn to a large scale, it was possible to determine for each temperature, time values necessary to produce F_o values from 0.5 to 20. These time values have been tabulated in Table 4. Every row in this table represents a set of times equivalent in terms of F_o. These times in combination with appropriate processing temperatures will result in the same sterilizing effect.

No equivalent times for RT = 225°F. were given for $F_o > 3$ because they would have been the result of extrapolation. The results agree well with expectations and the times required to produce the same F_o values are considerably longer at low temperatures than at higher temperatures. The same is true considering the differences in times corresponding to various levels of F_o within each temperature. The differences in processing times corresponding to the same two F_o values are considerably larger for low temperatures than for high processing temperatures, e.g., at 243°F. the processing time necessary to reach $F_o = 5$ is 14 minutes longer than the time resulting in $F_o = 1$, the respective difference for 315°F. is 2.25 minutes. The span for $F_o = 20$ and 1 is 49 minutes for 243°F. and only 4 minutes for 315°F. The decrease in the interval is even more evident when processing temperatures of 225°F. and 315°F. are considered. It requires 119.5 minutes to achieve $F_o = 3$ at 225°F. and only 18 minutes at 315°F.

The results of this investigation also indicate the existence of an extremely large variation in the sterilizing effect of the same thermal process applied to a number of containers. In spite of a very careful control of replicability of a thermal process in terms of its length and processing temperature where the variation of the contents of the can and of processing conditions (time and temperature) was held to a minimum, the calculated values of F_o (by the "General Method") for the same heat treatment

TABLE 4

Time (min.) necessary at each of six retort temperatures to reach equivalent F_o values at the center of the can

F_o	Retort temperature (°F.)					
	225	243	261	279	297	315
	Min.	Min.	Min.	Min.	Min.	Min.
0.5	64.0	43.0	31.0	20.0	19.0	15.00
1.0	74.0	49.0	34.0	23.0	21.0	16.50
1.5	90.0	53.0	36.0	25.0	23.0	17.00
2.0	102.0	55.0	37.5	26.0	24.0	17.50
2.5	111.0	57.0	38.5	26.5	24.5	17.75
3.0	119.0	59.0	39.5	27.5	25.0	18.00
3.5	.....	60.0	40.0	28.0	25.5	18.25
4.0	.....	61.5	41.0	28.5	26.0	18.50
5.0	.....	63.0	42.0	29.5	26.5	18.75
6.0	.....	65.0	43.0	30.5	27.0	19.00
10.0	.....	74.0	45.5	32.5	28.7	19.60
15.0	.....	88.0	47.4	34.1	30.0	20.20
20.0	.....	98.0	48.8	35.5	31.0	20.50

varied by a factor from 2 to 1000. The mean F_o values, however, displayed relatively smaller variation which made possible an interpretation of the effects of time and temperature on the sterilizing values at various locations in the container.

GENERAL

It is apparent that the interpretation and presentation of the results obtained during this study would gain in value, were the authors able to devise functional relationships (with an experimental error estimate) correlating the variables investigated. Instead, graphical methods were used and trends were estimated by visual evaluation. The authors do not consider visual evaluation of data as the proper method of interpretation of experimental results and the basis for drawing final conclusions. This method of evaluation should not be used in establishing fundamental relationships leading to postulation of a theory nor of a prediction equation to be used in process design or development because the degree of reliability of such evaluation is not well defined nor replicable; it may be used, however, as a first approximation for evaluation of general trends and as a rough guide in the prediction of results. Also it may be used as a source of information for the design of an investigation and determination of the size of sample to be used which would permit a more precise and rigorous statistical and mathematical treatment of data.

In the determination of F_o values during this investigation replicability, precision, and the number of determinations for each location in the can were not considered sufficient to warrant a rigorous statistical treatment of data, especially since the number of missing data was rather large. In shear force determination *(12)* similar variations were observed in spite of a much larger number of determinations (8 determinations of shear force to one of F_o value, and no missing data). The number of determinations of shear force justified, in the opinion of the authors, an attempt at evaluation of functional relationships by statistical methods but in view of the variations persisting in mean values the prediction equations established were termed only approximate. These prediction equations are of value because no theory is available to explain the behavior of shear force functions.

Progressing one step further in the determination of thermal characteristics *(7)*, definite prediction relationships were established with estimates of experimental error attached to them, because the number of determinations was large and the replicability of results seemed to be satisfactory. Furthermore, theoretical relationships were available for comparison of experimental values with theoretical expectations and with the values derived from the prediction equations developed. A rigorous statistical and mathematical treatment should be applied to data which warrant it, and the results of such treatment (within the limits of experimental error) can be used in postulating laws. The visual estimate cannot be used for this purpose, but may be of use in estimating trends when rigorous treatment is not justified by the quantity or quality of the experimental data.

SUMMARY AND CONCLUSIONS

An investigation was conducted of sterilizing effects in 300×308 cans of beef processed at higher than conventional retort temperatures (225-

315°F.) for short processing times (2-120 minutes). The distribution of F_o values at 25 locations within the container was established to provide data on sterilizing effect distribution in canned beef not available or scarce in the literature. Furthermore, information has been furnished on the effect of high processing temperatures for short times on the process lethality.

The contribution of the cooling phase, and especially that of residual heating during intermediate cooling, to the process lethality was ascertained and its effect on F_o distribution was evaluated. Both effects were found to be considerable.

The relationships among F_o values, processing times and temperatures were studied and trends were estimated by visual methods. Extremely large variations in the experimental results made it impossible to establish exact functional relationships. Log F_o was found to be a two-slope linear function of processing times for the six constant processing temperatures.

On the basis of the experimental results obtained during this investigation the following conclusions may be drawn:

(1) The distributions of F_o values display a doughnut-shaped volume of lowest values in the central horizontal section of the can rather than in the geometric center of the container. This effect is more pronounced at high temperatures.

(2) The effect of residual heating (during cooling) on the sterilizing effect and its distribution is considerable and as such should not be underestimated in thermal process determination.

(3) The F_o distributions observed are a secondary effect of distorted temperature distributions (caused by anisotropy of beef and initial temperature gradient in the can) and of residual heating.

(4) The F_o distributions observed emphasize the necessity of basing thermal process determination on the volume lethality concept.

The F_o determination is subject to great experimental variation. The size of the sample should be determined by statistical methods to insure determination of process resulting in a safe but not overprocessed product.

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DETERMINATION OF THE THERMAL DEATH RATE OF BACTERIA

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The problem of determining the conditions required to thermally sterilize a system is one of considerable practical and theoretical importance. Particular difficulties arise in those systems which undergo deterioration on being subjected to temperatures of the order required to destroy the more resistant spores. Under these circumstances, it becomes a problem of some importance to design a procedure which will bring about a desired level of sterilization without undue exposure of the sample to the destructive action of high temperatures.

Ball (*1*) published in 1928 certain methods generally employed in the food industries for computing thermal sterilization times. The work was reviewed and extended in 1949 by Stumbo (*5*). More recently Tischer and Hurwicz (*7*) have investigated the problem. The procedures as developed by Ball depend on an assumed logarithmic form of the heating curve and are thereby not applicable to heating curves which may be of a different form. Moreover, the required computations, though simple, are somewhat awkward from an arithmetical point of view since a series of preliminary quantities must be determined before the final computation of the processing time.

The procedures described in this communication provide a more generally applicable approach than those previously described. In addition, the solution of sterilization problems can be carried out in a more direct and arithmetically simpler manner by making use of certain tabulated functions. The present method has the further advantage that the contribution of each phase of a sterilization cycle can be independently evaluated.

THEORY

It has been established by a number of investigations (Rahn, *4*) that the thermal destruction of spores proceeds, in many instances, as a first order reaction, i.e.

$$\frac{dN}{dt} = ZN \tag{1}$$

where N is the concentration of the organism per unit of material and Z is the rate constant for thermal inactivation of the organism. The constant, k, has been expressed by Glasston *et al.* (*2*) in the form

$$Z = \left(\frac{k}{h} Te \frac{\Delta S^*}{R}\right) e^{-\Delta H^*/RT} \tag{2}$$

where ΔS^* is the standard entropy of activation; ΔH^* the standard enthalpy of activation; T the absolute temperature and h, k, R, Plank's constant, Boltzmann's constant, and the gas constant, respectively. Formulation of the thermal death processes

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of bacteria in terms of the concepts implied by equation (2) carries with it the concept of a free energy barrier which must be surmounted by certain critical systems within the bacteria (enzymes, nucleoproteins, etc.) in order that inactivation can occur. Only those systems with a free energy greater than or equal to that of the barrier will undergo denaturation.

Over a sufficiently small temperature range, the entropy term may be regarded as a constant, A, and may be written in a somewhat simpler manner

$$Z = Ae^{-B/T} \tag{3}$$

where B is a constant proportional to the experimentally determined activation energy (B=E/R). Equation (3) is in the form of the well known Arrhenius equation. The assumption is next made that equation (3) represents the rate of thermal inactivation of spores. Equation (1) can now be written in integrated form as

$$\frac{2.30}{t} \log \left(\frac{N_o}{N}\right) = Ae^{-B/T} = Z \tag{4}$$

where N_o is the initial concentration of the spores and N the concentration at time t when the system has been subjected to a temperature T.

At any two temperatures, T_1 and T_2 equation (5) permits the calculation of the constants:

$$\log A = \frac{T_2 \log Z_2 - T_1 \log Z_1}{T_2 - T_1} \tag{5}$$

$$B = 2.30\, T_1\, T_2 \frac{(\log Z_2 - \log Z_1)}{T_2 - T_1} \tag{6}$$

According to equation (6) the slope obtained by plotting log Z vs T is not a constant because of the factor, $T_1\, T_2$. However, over the temperature range of interest in sterilization processes, 180° F. to 270° F. (356°T to 405°T), the factor varies at the most by 10% or so which is within the usual experimental error for determining Z. Because of this relatively small variation, equation (6) can be written in terms of the mean absolute temperature, T_m, over the interval as

$$B = 2.30\, T_m^2 Z^1 \tag{7}$$

where Z^1 is the slope term of (6). (The symbol Z is usually reserved for the reciprocal of Z^1.) Equation (7) enables the calculation of B from a knowledge of Z^1.

In order to test the validity of the assumption that the thermal death rate can be represented by equation (3) in the case of the more resistant spores, the date reported by Stumbo *et al.* (6) for the organism, Putrefactive Anaerobe 3679 (National Canner's Association strain) in pureed peas has been analyzed. Applying equations (5) and (6) to these data gives the result

$$B = 3.65 \times 10^4 \text{ and } A = 3.98 \times 10^{40}$$

The computed values are compared with the experimental data in Table 1. Though the computed values tend to run somewhat high, the agreement is sufficiently close to warrant using the above values of A and B in the analysis given below.

TABLE 1

Comparison of experimental and calculated death rates for P.A. 3679.

Temp (abs.)	Temp. (°C.)	Z (exp.)	Z (cal.)
377	104	.0340	.0398
383	110	.122	.173
388	115	.441	.501
394	121	1.51	2.51
399	126	6.63	7.95
405	132	26.8	31.0

It is interesting to note that similar calculations with the data of Stumbo *et al.* (*6*) for the spores of *Clostridium botulinum* in pureed peas give the value $B = 4.15 \times 10^4$. This value is greater than that for P.A. 3679 despite the fact that the latter is more resistant to thermal inactivation by heat. All other things being equal, equation (3) would imply that the larger the B value the more resistant the organism. The anomaly is resolved on computing the A value for *Cl. botulinum* which gives $A = 1.86 \times 10^{48}$. This latter value is considerably greater than that of P.A. 3679. In terms of rate process theory, as expressed by 2, this result means that the greater rate of destruction of the *Cl. botulinum* spores is due to the larger standard entropy of activation since,

$$A = \frac{k}{h} Te \frac{\Delta S^*}{R} \tag{8}$$

Equation (8) permits an estimation of the entropies of activation:

ΔS^* (*Cl. botulinum*) = 160 entropy units
ΔS^* (P.A. 3679) = 123 entropy units

Since an increase of entropy is associated with an increased structural disorganization of a system, the above analysis would seem to imply that the thermal inactivation of the critical systems in *Cl. botulinum* involves a greater degree of over-all disorganization than is the case with P.A. 3679.

The thermal sterilization condition. The above analysis leads directly to the conditions which must be satisfied in order that a sample be sterilized. The thermal sterilization time, t_s, may be defined as that time of sterilization which when just exceeded results in a spore concentration of less than one, that is, all the spores are just destroyed; therefore, from (4)

$$t_s = \frac{2.30}{Z} \log (N_o) \tag{9}$$

Since the number of spores varies from sample to sample, the question arises as to a proper choice of N_o, the initial concentration of spores. This choice depends, ultimately, on the statistical character of the spore distribution in the sample. If it is assumed that the distribution follows the normal law with an average value $\overline{N}_o$ and a standard deviation σ, then it is possible to estimate the risk of failing to sterilize a sample. Let

$$N_o = \overline{N}_o + n\sigma,$$

where n is a number arbitrarily chosen depending on the risk one is willing to assume that the sample will contain a spore concentration greater than N_o. If $n = 4$, then the probability of finding a sample with a spore concentration greater than $\overline{N}_o + 4\sigma$ is about three in a hundred thousand, while for $n = 5$ it is about three in ten million. The sterilization time depends, then, on Z, $\overline{N}_o$ and σ and is given by

$$t_s (Z, \overline{N}_o, \sigma) = \frac{2.30}{Z} \log 10 (\overline{N}_o + n\sigma) \tag{10}$$

It is clear from this expression that the greater the n level, the greater the sterilization time must be. In practice, a compromise must be achieved between the risk of failing to sterilize a sample and the increased deterioration due to heating.

In practice, a sample is brought from room temperature to some fixed temperature and then cooled. In order to minimize the exposure of the sample to heat it is desirable to consider the sterilizing action of all phases of the cycle. During variable temperature processes, the condition for sterilization is, from (9) and (10)

$$A \int_o^t e^{-B/T} dt \geqq 2.30 \log (\overline{N}_o + \sigma n) \tag{11}$$

where t is the time of sterilization. The minimal time is obtained by choosing the equality condition. The temperatures employed in these calculations are those at the region in the sample of greatest temperature lag, since at this region the time of survival is the longest. Figure 1 represents some rather typical data for conductive heating of

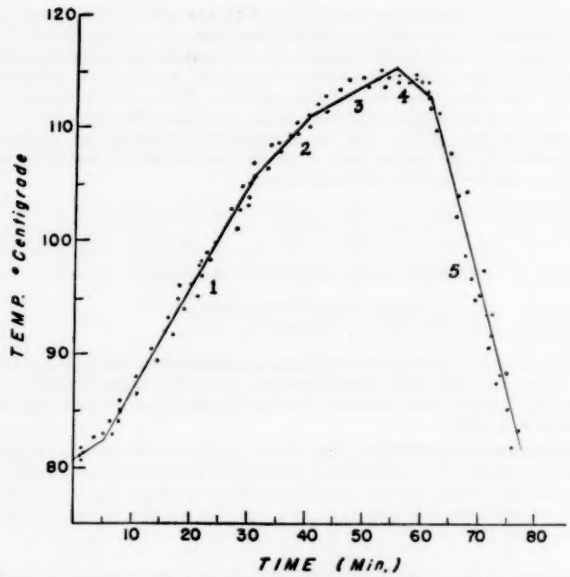

Figure 1. A representative heating curve indicating the disposition of typical experimental values (.) and the approximating linear expressions (——).

container. Over any region of this curve it is possible to approximate the data by a set of linear relationships.

$$T = a_i t + b_i$$

where a_i represents the slope of the approximating line over some region. A set of such approximating lines is shown in Figure 1. By choosing a sufficient number of linear relationships it is possible to represent the data as closely as is desired. These lines may be obtained, in the most accurate cases, by means of least squares or, when less accuracy is needed, by simple graphical inspection. The linear relationship permits (11) to be written, for all linear paths,

$$A\Sigma_i \frac{1}{a_i} \int_{T_2}^{T_1} e^{-B/T}\, dT = A\Sigma_i \frac{\lambda_i (T_1T_2)}{a_i} = 2.30 \log (\overline{N}_o + \sigma N) \qquad (12)$$

The function $\lambda\ (T_1T_2)$ is referred to as the thermal sterilization function (TSF) between the limits T_1 and T_2.

Computation of the thermal sterilization function. The result permits a convenient computation of the thermal sterilization condition, since the entire procedure consists, simply, of adding $\lambda(T_1T_2)/a_i$ so that sum equals the logarithmic value on the right. Any combination of circumstances which satisfies this condition will result in sterilization. The computation can be made with considerable ease from a tabulated value of TSF.

The integration of the (TSF) can be carried out by the usual methods by making the transformation $1/T = u$ so that

$$\lambda (T_1T_2) = \int_{u_1}^{u_2} \frac{e^{-Bu}}{u^2}\, du = \left[\frac{e^{-Bu}}{u} + B \left\{ \log u + \sum_{N=1}^{\infty} \frac{(-11^N (Bu)^N}{n\,.\,n!} \right\} \right]_{u_1}^{u_2} \qquad (13)$$

However, the power series on the right converges very slowly for values of (Bu) in the region of interest ($B_u = 100$). It is better, therefore, to proceed in a somewhat different manner. The integral on the left-hand side (13) may be written

$$\int_{u_1}^{u_2} \frac{e^{-Bu}}{u^2}\,du = \left[\frac{e^{-Bu}}{u}\Bigg|_{u_1}^{u_2} + B\left(\int_{u_1}^{u_2} \frac{e^{-Bu}}{u}\,du\right)\right] \tag{14}$$

The integral on the right-hand side of equation (14) for $Bu >> 1$ is best evaluated by means of an asymtotic expansion,

$$\int_{u_1}^{u_2} \frac{e^{-Bu}}{u}\,du = -\frac{e^{-Bu}}{Bu}\left[1 + \sum_{N=1}^{\infty} \frac{(-1)^N N!}{N!\,(Bu)}\right]_{u_1}^{u_2} \tag{15}$$

The power series so obtained converges very rapidly. Substituting (15) into (14) there results

$$\lambda\,(T_1T_2) = \frac{e^{-Bu}}{u}\,\Sigma\left(\frac{(-1)^N N!}{(Bu)^n}\right)\Bigg|_{u_1}^{u_2}$$

Because of the rapid convergence of the power series only the first two terms need be considered in most applications, so that reverting to the original notation there finally results

$$\lambda\,(T_1T_2) = \left|\,Te^{-B/T}\left(\frac{T}{B} - 2\left(\frac{T}{B}\right)^2\right)\right|_{T_1}^{T_2} \tag{16}$$

Table 2 has been computed for P.A. 3679 ($B = 3.65 \times 10^4$) over 5° C. intervals and is given for purposes of illustration. Equation (16) permits computation of TSF over any interval and for any degree of subdivision of the interval of choice.

Computation of sterilization conditions. The application of the method described here involves the use of the equations,

$$\frac{2.30}{A}\log\left(\overline{N}_o + \sigma n\right) = \Sigma_i \frac{\lambda\,(T_1T_2)}{a_i}$$

$$a_i = \frac{\Delta T\ (°C)}{\Delta t\ (min)}$$

and a table of TSF (Levine 1954) for the particular organism under the particular set of conditions of interest. If the heating data are that of Figure 1 and $\overline{N}_o = 10^6$ while $\sigma = 10^2$,

TABLE 2

Thermal sterilization functions for P.A. 3679 in pureed peas

$10^{10} \times \lambda\,(T_1\ T_2)$

°C	82	87	92	97	102	107	112	117	122	127
87	2.66x10-4	—								
92	1.33x10-3	1.06x10-3	—							
97	6.68x10-3	6.31x10-3	5.35x10-3	—						
102	2.82x10-2	2.79x10-2	2.69x10-3	2.15x10-2	—					
107	.102	→	.101	.096	.074	—				
112	.337	→	→	.331	.309	.235	—			
117	1.08	→	→	→	1.05	.980	.743		—	
122	3.85	→	→	→	3.82	3.75	3.51	2.77		
127	12.0	→	→	→	→	11.9	12.1	10.9	8.1	
132	35.6	→	→	→	→	35.5	35.3	34.5	31.7	23.6

then all data required for the analysis of the problem are on hand. The data (Fig. 1) can be approximated by dividing the points into groups and drawing a straight line so that the points over a particular region scatter equally on both sides of the line. The slopes of each line then can be read immediately for each temperature interval. In the case illustrated, that of slow conductive heating, the curve has been divided into five linear regions with slopes

$$
\begin{aligned}
(\ 82^\circ \text{ C.} - 105^\circ \text{ C.})\ a_1 &= .92 \\
(105^\circ \text{ C.} - 111^\circ \text{ C.})\ a_2 &= .60 \\
(111^\circ \text{ C.} - 115^\circ \text{ C.})\ a_3 &= .26 \\
(115^\circ \text{ C.} - 112^\circ \text{ C.})\ a_4 &= .50 \\
(112^\circ \text{ C.} - \ 82^\circ \text{ C.})\ a_5 &= 2.5
\end{aligned}
$$

In the case of more rapid heating, such as that occurring when convection or agitation play a role in heat transport, the curvature associated with the data will be less and fewer linear relationships required. Under these, as well as under other circumstances, the heating data may deviate from the sort of approximations employed by Ball so the application of the earlier procedure is dubious or invalid. It should be noted in passing that the method discussed here deals directly with the data while the earlier procedure requires that temperature be plotted as the logarithm. Since relatively large variations in the temperature will produce much smaller changes in the logarithm of the temperature, Ball's method suffers from an inherent inaccuracy which is avoided here.

When the requirements are such that a great number of samples need to be sterilized, n may be chosen as 5 so that the chance of a spore concentration greater than $10^5 + 510^4$ (105,000) is about less than three in ten million. This method of choosing the spore level has the advantage over a purely arbitrary choice, such as suggested by Stumbo (*5*), in that one works directly with the spore distribution and at a known probability level. The condition for sterilization is then (for P.A. 3679)

$$\frac{2.30}{3.98} \log (1.0510^5) = 2.91$$

Dividing each TSF (Levine, *3*) by the corresponding slope there results

$$
\begin{aligned}
\frac{\lambda\,(83, 105)}{a_1} &= .067 \\
\frac{\lambda\,(105, 111)}{a_2} &= .322 \\
\frac{\lambda\,(111, 115)}{a_3} &= .600 \\
\frac{\lambda\,(112, 115)}{a_4} &= .150 \\
\frac{\lambda\,(82, 112)}{a_5} &= \frac{.135}{1.274}
\end{aligned}
$$

Since this sum is less than 2.91 it is clear that sterilization at the 5σ level is not achieved by the cycle. A further advantage of the method is made apparent by these calculations, namely, the contribution of each portion of the heating cycle is separately evaluated. This enables independent study of each phase of the sterilization process.

The necessary alteration in the heating cycle which will result in sterilization can be readily computed. If it is desired to increase the sterilization by, say, increasing the cooling time over the interval 112° C. to 82° C., keeping all other rates the same, then we seek a time such that

$$
\begin{aligned}
\frac{\lambda\,(82, 112)}{\Delta T\ (^\circ\text{C.})}\ \Delta t &= 2.91 - 1.13 \\
\Delta T\ (^\circ\text{C.}) &= 30^\circ \text{ C.}
\end{aligned}
$$

therefore

$$\Delta t = 94.5 \text{ min.}$$

A number of computations of the type illustrated above can be performed, and it is apparent that these computations are both simple and straightforward once a Table TSF has been prepared for the particular organisms and conditions that are of interest.

SUMMARY

The kinetics and thermodynamics of spore inactivation by heat are discussed. The resulting analysis is applied to the thermal inactivation of P.A. 3679 and *Clostridium botulinum*. Certain essential differences in the energetics of the thermal inactivation processes in the two instances are emphasized.

A general method is developed for determining the conditions for thermal sterilization of samples by means of thermal sterilization functions.

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CHARACTERISTICS OF SHORTENED CAKE BAKED IN A FAST- AND IN A SLOW-BAKING PAN AT DIFFERENT OVEN TEMPERATURES[a]

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Kind of metal used and its finish are of importance in baking pans. These variables affect not only baking time but also volume, shape of the top, degree and evenness of browning, and quality of the crumb of shortened cake, Charley *(1)*. Shortened cakes baked in a steel, a japanned iron, an anodized aluminum, or a sheet iron pan—all either dark or dull metals—reached a maximum internal temperature in significantly less time than did cakes baked in pans made of bright aluminum, tinned iron, stainless steel, or copper. At a temperature of 365°F., pans classified in the first, i.e., dark or dull, group yielded cakes in which there was a greater tendency for the tops to hump and crusts that were both browner and less evenly browned than was the case with the pans of bright finish. But cakes with larger volumes and with higher rating over-all crumb quality were yielded by the first group.

The present work was undertaken to see whether there would still be differences in shortened cake baked in a fast- and in a slow-baking pan at oven temperatures so adjusted that the baking times were identical.

EXPERIMENTAL

Baking pans. Two small loaf pans, $5 \times 3 \times 2\frac{3}{4}$ in., from the previous work *(1)* were used, one the fast-baking, dark japanned iron pan and the other the slow-baking, bright tinned iron pan.

Cake batter. Formula for the cake batter and the method of mixing was the same as those used in the previous work *(1)*. Two hundred twenty-five grams of batter were baked in each pan.

Adjustment of baking temperatures. Because an oven temperature of 365°F. had been used in the previous work with cake pans, that temperature was used as a basis of comparison for both the japanned and the tinned iron pan. Preliminary trials showed that cakes baked in the japanned iron pan at an oven temperature of 345°F. would reach a maximum internal temperature in the same length of time, 27 minutes, as did cakes baked in the tinned iron pan at an oven temperature of 365°F. Cakes in the tinned iron pan baked at an oven temperature of 395°F. reached a maximum internal temperature in the same length of time, 24 minutes, as did cakes baked in the japanned iron pan at an oven temperature of 365°F. In all cases cakes were baked for 2 minutes beyond the time required to reach maximum internal temperature. There were 8 replications for each of the 2 paired sets of pan-temperature combinations.

Cake volume. Using a miter box, a slice was cut from each end of each cake and a third cut was made crosswise through the center. Tracings were made around each of the end slices and around the facing edges of the cut through the center of the cake. The area in square inches of each of the 4 tracings was measured with a polarizing planimeter *(2)*. The average in square inches of the 4 areas was taken as the standing

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index to volume of the cake. The difference between the average area of the tracings of the 2 center cuts and the average area of the tracings of the 2 end slices was taken as an index of the extent of humping of the tops.

Scoring the cakes. Paired slices of cakes from the 2 pans baked at each of the 2 sets of temperatures were presented to a panel of 5 judges who were asked to indicate which cake in each pair was superior for each of the items listed on the score card. Included were fineness of cells, velvetiness of texture, moistness, tenderness, and overall desirability.

RESULTS AND DISCUSSION

Volumes. The standing index to the volumes of the cakes baked in the 2 pans at the 2 sets of temperatures are given in Table 1. With a baking

TABLE 1

Standing index to volume of shortened cakes baked in a fast- and in a slow-baking pan[1]

Type of pan	Baking time	Oven temp.	Standing index to volume
	Minutes	°F.	Square inches
Japanned iron	27	345°	6.67
Tinned iron	27	365°	6.39
Japanned iron	24	365°	6.64
Tinned iron	24	395°	6.52

[1] Based on 8 replications.

time of 27 minutes, the mean of the differences between the index to volume of the series of cakes from the two pans was 0.28 sq. in. and this difference was highly significant ($t = 35.0$ with 7 degrees of freedom). With a baking time of 24 minutes, the mean of the differences between the index to volume of the series of cakes from the 2 pans was 0.15 sq. in. and again the difference was highly significant ($t = 18.0$ with 7 degrees of freedom). In both instances, when the temperature was elevated for the slower-baking pan to shorten the baking time to that of the faster-baking pan, cakes from the latter still were larger in volume. Thus, altering the oven temperature to this extent did not compensate for the effect on the volume of the cake of the material from which the cake pan was made.

Shape of the top of cakes. The index to the shape of the tops of the cakes from each of the 2 pans at the 2 sets of temperatures is given in Table 2. The mean of the differences between the index to the shape of the tops of paired cakes baked in the japanned iron pan at 345°F. compared with those baked in the tinned iron pan at 365°F. was 0.19 sq. in. This difference was highly significant ($t = 11.93$ with 7 degrees of freedom).

TABLE 2

Index to shape of the tops of cakes baked in a fast- and in a slow-baking pan[1]

Type of pan	Baking time	Baking temp.	Differences in index to volume of center cut and end cut
	Minutes	°F.	Square inches
Japanned iron	27	345°	1.60
Tinned iron	27	365°	1.41
Japanned iron	24	365°	1.74
Tinned iron	24	395°	1.93

[1] Based on 8 replications.

Thus, with this pair of temperatures and with a baking time of 27 minutes, cakes baked in the faster-baking pan at the lower temperature humped more than did those baked in the slower-baking pan at the higher temperature.

The mean of the differences between the index to the shape of the tops of the paired cakes baked in the japanned iron pan at 365°F. and in the tinned iron pan at 395°F. was 0.21 sq. in. This difference was highly significant ($t = 17.62$ with 7 degrees of freedom). With this pair of temperatures and with a baking time of 24 minutes, the cakes baked in the slower baking pan at the higher temperature humped more than did those in the faster-baking pan at the lower temperature. Apparently, the higher baking temperature (395°F.) contributed to greater humping of the cakes by speeding the setting of the exposed upper surface of the cake batter. With both pairs of temperatures, the crack in the top was more pronounced in those cakes from the tinned iron pan at the higher temperature than in those from the japanned iron pan at the lower temperature.

Browning of the crust. Cakes baked in the japanned iron pan at 345°F. had somewhat lighter top crusts and definitely browner side and bottom crusts than did cakes baked in the tinned iron pan at 365°F. Cakes baked in the japanned iron pan at 365°F. had decidedly lighter top crusts and slightly darker side and bottom crusts than did cakes baked in the tinned iron pan at 395°F. Cakes from the tinned pan were more evenly browned and elevating the temperature for the japanned iron pans only accentuated the uneven browning of cakes from this pan.

Crumb quality. A summary of the ranks of the cakes by the judges is included in Table 3. The judges selected the cake from the japanned iron

TABLE 3

First rankings for paired cakes from two pan-temperature combinations[1]

Characteristics	Pan-baking temperature combinations				Total		X^2 value[2]
	Japanned at 345° F.	Tinned at 365° F.	Japanned at 365° F.	Tinned at 395°F.	Japanned	Tinned	
Fineness of cells	25	15	24.5	15.5	49.5	30.5	4.51
Texture	27.5	12.5	25.5	14.5	53	27	8.50
Moistness	23	17	22	18	45	35	1.25
Tenderness	27	13	21.5	18.5	48.5	31.5	3.61
Over-all desirability	26.5	13.5	25	15	51.5	28.5	6.62

[1] 5 judges and 8 replications for each pan-temperature pair.
[2] X^2 value > 3.841 is significant.

pan at the lower temperature as superior more frequently than the cake from the tinned iron pan at the higher temperature. However, on the basis of the chi-square test, the former were judged superior a significantly greater number of times for fineness of cells, for texture, and for over-all desirability, but not for moistness or for tenderness.

SUMMARY

The effects on quality of shortened cake of adjusting the baking temperature for a fast-baking japanned iron pan and for a slow-baking tinned iron pan to give baking times of identical length were investigated. The

baking time was 27 minutes for cakes baked in a japanned iron pan at 345°F. and in a tinned iron pan at 365°F. The baking time was 24 minutes for cakes baked in a japanned iron pan at 365°F. and in a tinned iron pan at 395°F.

Elevating the baking temperature for the tinned iron jan produced a cake of less volume than that baked in the japanned iron pan at the lower temperature. With a baking time of 27 minutes, humping of the top was more pronounced in cakes from the japanned iron pan. With a baking time of 24 minutes, it was more pronounced in cakes from the tinned iron pan. Cakes baked in the faster-baking pan were less evenly browned, but those from the slower-baking pan had a more pronounced crack in the top. Cakes baked in the faster-baking pan had finer grain, more velvety texture, and higher over-all crumb quality rating than did cakes from the slower-baking pan. For moistness and for tenderness the judges failed to select the cake from the faster-baking pan a significantly greater number of times.

It is concluded that with the two metal cake pans used the temperature adjustments investigated failed to compensate for the effects on shortened cake of the baking pan material.

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STUDIES IN THE DEEP-FAT FRYING OF CASHEW KERNELS

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An account of commercial practices in processing and storing cashew nuts and kernels has been recently provided by Kapur, Prasad, and Mathur *(3)*. During the 10 months ending January 1955, 577,013 tons of cashew kernels valued at Rs. 93,229,000 ($19,668,500) were exported from India to various foreign countries including U. S. A., U. K., Canada, and Australia *(5)*. Export trade in cashew kernels is confined mainly to raw kernels.

However, certain factories in India are canning roasted and salted cashew kernels for home consumption and limited export. The industry uses liquid paraffin as the roasting medium and the storage life of the roasted kernels is 3-4 months. Two methods for this purpose have been developed at this Institute, one involving roasting and the other deep-fat frying in "vanaspati" and the storage lives of the ready-to-serve kernels when canned under ordinary atmosphere have been found to be 6 and 12 months, respectively *(7, 8, 9)*.

The present paper reports data obtained in connection with the deep-fat frying of cashew kernels including a study of the deterioration of the frying media.

MATERIAL AND METHODS

Raw cashew kernels belonging to grade 320 (i.e. the weight of 320 kernels was approximately 1 lb.) were obtained from a well-known firm in Calicut. This firm packs raw cashew kernels in kerosine-type cans, each holding 25 lbs. After filling, the cans are exhausted and subsequently filled with carbon dioxide. Under these conditions of packing, the kernels have been found to remain in an excellent condition for at least one year. Such CO_2-packed kernels were used for the experiments described in this paper.

Two frying media, viz., refined peanut oil and "vanaspati" (hydrogenated peanut oil with 5% sesame oil added for easy detection of adulteration) were used. It was noticed that after each frying operation there was a fall of 15-20°C. in the temperature of the oil. This applied both to refined peanut oil as well as "vanaspati." Hence in all frying operations care was taken to see that the oil regained its original temperature before immersing in it a subsequent lot of kernels.

Moisture contents in cashew kernels were determined by heating 3 g. finely chopped cashew kernel tissue at 130 ± 2°C. for 2 hours. These conditions for the determination of the moisture content were standardised after a preliminary experiment. Acidity was estimated according to the method described by Jamieson *(2)* and the peroxide value by the Wheeler's method *(10)*.

RESULTS

Details regarding the deep-fat frying of cashew kernels in refined peanut oil are provided in Table 1. At all the 3 frying temperatures, viz., 160, 170, and 180°C., the percentages of moisture lost during frying increased with increasing durations of frying. On the other hand, the percentages of gain in weight at the end of frying operations decreased with increasing durations of frying. As a result of these opposing tendencies, the oil uptake tended to be the same at all the frying temperatures and with respect

TABLE 1

Details regarding the deep-fat frying of cashew kernels in refined peanut oil (200 g. lots of kernels were deep-fried successively in one lot of 3 lb. of oil)

Lot No.	Frying		% moisture					% gain in weight of kernels		% oil-uptake		% excess oil removed as a result of centrifuging	Organoleptic condition of fried kernels
	Temp., °C.	Duration, secs.	In raw kernels	In fried kernels		Lost during frying operations							
				A[1]	B[2]	A[1]	B[2]	A[1]	B[2]	A[1]	B[2]		
1	160	60	5.70	4.21	.....	1.49	.....	1.90	.....	3.39	.....	.....	Underfried
2	160	70	5.70	4.00	3.91	1.70	1.79	1.49	0.90	3.19	2.69	0.50	Slightly underfried
3	160	80	5.70	3.67	3.72	2.03	1.98	1.09	0.75	3.12	2.73	0.39	Good
4	160	90	5.70	3.51	.....	2.19	.....	0.99	.....	3.18	.....	.....	Slightly overfried
5	170	45	5.70	4.35	.....	1.35	.....	1.90	.....	3.25	.....	.....	Underfried
6	170	55	5.70	3.48	.....	2.22	.....	1.29	.....	3.51	.....	.....	Underfried
7	170	65	5.70	3.30	3.10	2.40	2.60	1.20	0.80	3.60	3.40	0.20	Good
8	170	75	5.70	2.86	2.70	2.84	3.00	1.00	0.68	3.84	3.68	0.16	Good
9	180	40	5.70	3.94	.....	1.76	.....	1.54	.....	3.30	.....	.....	Underfried
10	180	50	5.70	3.23	3.20	2.47	2.50	1.32	0.70	3.79	3.20	0.59	Good
11	180	60	5.70	3.00	2.95	2.70	2.75	0.69	0.50	3.39	3.25	0.14	Slightly overfried
12	180	70	5.70	2.80	.....	2.90	.....	0.00	.....	2.90	.....	.....	Overfried

[1] A—after draining.
[2] B—after draining and centrifuging.

TABLE 2

Details regarding the deep-fat frying of cashew kernels in "vanaspati" (200 g. lots of kernels were deep-fried successively in one lot of 3 lb. of oil)

Lot No.	Frying		% moisture					% gain in weight of kernels		% oil-uptake		% excess oil removed as a result of centrifuging	Organoleptic condition of fried kernels
	Temp., °C.	Duration, secs.	In raw kernels	In fried kernels		Lost during frying operations							
				A[1]	B[2]	A[1]	B[2]	A[1]	B[2]	A[1]	B[2]		
1	160	60	5.70	.....	3.50	.....	2.20	.....	−0.25	.....	1.95	.....	Underfried
2	160	70	5.70	3.45	3.40	2.25	2.30	0.50	−0.75	2.75	1.55	1.20	Good
3	160	80	5.70	3.40	3.20	2.30	2.50	0.50	−0.98	2.80	1.52	1.28	Excellent
4	160	90	5.70	.....	2.90	.....	2.80	.....	−1.47	.....	1.33	.....	Slightly overfried
5	170	45	5.70	.....	3.60	.....	2.10	.....	−0.92	.....	1.18	.....	Underfried
6	170	55	5.70	.....	3.40	.....	2.30	.....	−1.19	.....	1.11	.....	Underfried
7	170	65	5.70	3.40	3.30	2.30	2.40	0.15	−1.53	2.45	0.87	1.58	Good
8	170	75	5.70	3.35	2.80	2.65	2.90	0.25	−1.80	2.90	1.10	1.80	Good
9	180	40	5.70	.....	3.50	.....	2.20	.....	−0.90	.....	1.30	.....	Underfried
10	180	50	5.70	3.30	3.40	2.40	2.30	−0.70	−1.70	1.70	0.60	1.10	Good
11	180	60	5.70	2.75	2.70	2.95	3.00	−0.95	−2.00	2.00	1.00	1.00	Slightly overfried
12	180	70	5.70	.....	2.15	.....	3.55	.....	−2.25	.....	1.30	.....	Overfried

[1] A—after draining.
[2] B—after draining and centrifuging.

to all the durations of frying. Judged from the results of organoleptic tests in each of which a panel of 6 judges was employed, a temperature of 160°C. and a duration of 80 seconds are considered as the optimum conditions for the frying of cashew kernels in peanut oil. Under these conditions of frying, the oil-uptake was of the order of 2.73%. Essentially similar data were obtained in another experiment where "vanaspati" was used as the frying medium (Table 2). Optimum conditions for frying were found to be the same as for refined peanut oil, but the oil-uptake was smaller, being of the order of 1.52%.

Another experiment was conducted using "vanaspati" as the frying medium and the temperature of frying was maintained at 160°C. In this experiment, the duration of frying was varied from 50 seconds to 270 seconds and the proportion of kernels to fat was also varied, 114 g. and 228 g. cashew kernels being fried in 3 lbs. of the frying medium. The results are graphically shown in Figures 1 and 2. It is seen from Figure 1 that moisture loss from kernels progressively increases with increasing durations of frying. Moreover, the moisture loss is greater if the proportion of kernels with respect to a given quantity of oil is reduced. Figure 2 illustrates that oil-uptake of the kernels remains more or less constant over a wide range of durations of frying, the oil-uptake being greater if the proportion of kernels with respect to a given quantity of oil is reduced.

In the third experiment the comparative deterioration of peanut oil and "vanaspati" during frying of cashew kernels was studied. In both the cases, the temperature of frying was maintained at 160°C. and the duration of frying was 80 seconds. The results are presented in Table 3. It was found that refined peanut oil started to give off undesirable odour after frying 200 g. cashew kernels whereas "vanaspati" was without undesirable odour even after frying 11,000 g. cashew kernels. Porter, Michaelis, and Shay *(6)* state that the percentage of free fatty acid present in a used fat is a fairly reliable measure of the extent of its breakdown and of its objectionable quality of imparting a poor flavour to food fried therein.

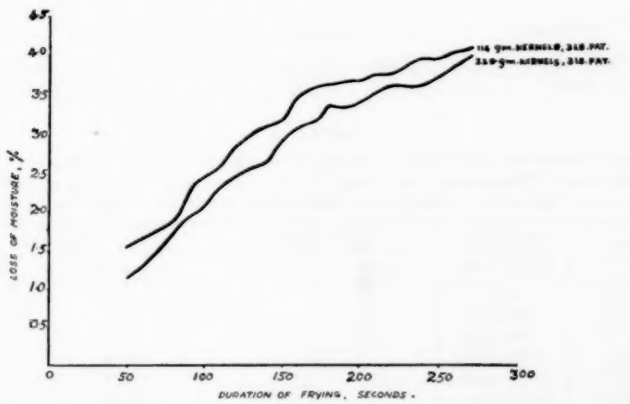

Figure 1. Relation between duration of frying and loss of moisture from cashew kernels (temperature of frying, 160° C.).

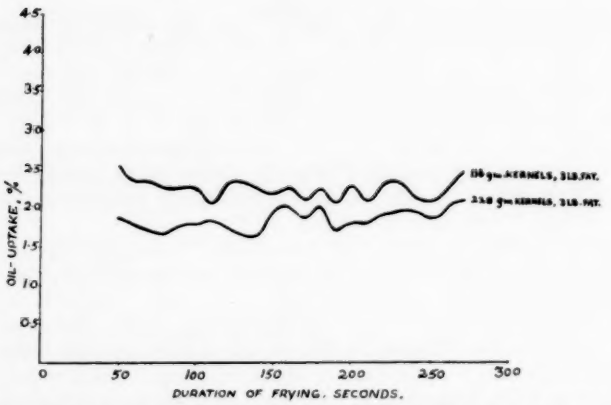

Figure 2. Relation between duration of frying and oil-uptake of cashew kernels (temperature of frying, 160°C.).

They also state that used fats with above 2% acid (as oleic) may impart an objectionable flavour to the foods fried in them. In our experiments the deterioration during frying was found to be far greater in the case of peanut oil as compared to "vanaspati." Macleod and Nason *(4)* state that fats hardened by hydrogenation have little tendency to become rancid by hydrolysis and may be heated to high temperatures without decomposition. They also point out that in deep-fat frying there is danger of decomposition unless care is exercised to prevent overheating. Once the fat has commenced to decompose, the acid formed hastens further decomposition. According to Blunt and Feeney *(1)*, the approximate temperatures of decomposition for peanut oil and hydrogenated fats are 150-160° C. and 219-232°C., respectively. From these considerations, "vanaspati" should be considered a better frying medium than refined peanut oil.

SUMMARY

A study of the factors involved in the deep-fat frying of cashew kernels has been made. A temperature of 160°C. and a duration of 80 seconds

TABLE 3

Comparative deterioration of peanut oil and "vanaspati" during the deep-fat frying of cashew kernels (Temperature of frying, 160°C.; duration of frying, 80 seconds; 200 g. lots of kernels were deep-fried successively in one lot of 4.25 lbs. of oil)

Date of frying	Cumulative quantities of kernels fried	Refined peanut oil		"Vanaspati"	
		Acidity[1]	P.V.[2]	Acidity[1]	P.V.[2]
11-6-53	Nil	0.30	11.7	0.09	1.7
11-6-53	1000 gms.	0.36	95.9	0.11	10.6
11-6-53	2000 gms.	0.39	130.5	0.11	17.8
11-6-53	3000 gms.	.....	.....	0.11	20.1
11-6-53	4000 gms.	.....	.....	0.12	20.9
12-6-53	11,000 gms.	.....	.....	0.28	24.0

[1] Percentage oleic acid.
[2] Milliequivalents of peroxide oxygen per kilogram of oil.

have been found to be the optimum conditions for the deep-fat frying of cashew kernels. The percentages of moisture lost during frying of cashew kernels increased with increasing durations of frying. On the other hand, the percentages of gain in weight of kernels at the end of frying operations decreased with increasing durations of frying. As a result of these opposing tendencies, the oil-uptake tended to be the same at all the frying temperatures and with respect to all the durations of frying investigated. It was found that the moisture loss from the kernels is greater if the proportion of kernels with respect to a given quantity of oil is reduced. On the other hand, the oil-uptake by the kernels increased when the proportion of kernels with respect to a given quantity of oil was reduced. The deterioration is far greater in the case of peanut oil as compared to "vanaspati" during the frying of cashew kernels.

Acknowledgment

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THE EFFECT OF TWO METHODS OF COOKING ON PALATABILITY SCORES, SHEAR FORCE VALUES, AND COLLAGEN CONTENT OF TWO CUTS OF BEEF

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Suiting the method of cooking to steaks which are available so that the cooked meat may be tender and palatable is still a problem and one of great practical importance. Steaks from rib and loin cuts are classed as "tender" because a small amount of connective tissue is said to be present *(11, 12, 18)*. Steaks from the round are among those classed as "less tender" because a larger amount of connective tissue is said to be present. Dry heat methods have been recommended for the tender cuts but, to obtain satisfactory tenderness in the less tender cuts, moist heat methods have been recommended *(3)*.

From a theoretical point of view, Cover *(6)* doubted that moist heat, in the sense of added moisture, was necessary to make meat tender. She cooked top and bottom round roasts to an internal temperature of 80°C. in water and in an oven of the same temperature (90°C.). The roasts were more tender by the dry heat method. Later Cover and Shrode *(5)* used moist and dry heat methods standardized to correspond as nearly as possible to the moist and dry heat methods used in many homes. Using these methods with tender and less tender cuts from four animals, they found that well-done bottom round steaks were more tender braised than broiled but that in well-done loin steaks the difference in tenderness was much less marked. Whether or not this was a result of the smaller amount of collagen in the loin steaks was not determined.

The aim of this study was to obtain a clearer understanding of what happens in the meat under practical cooking conditions involving moist and dry heat.

EXPERIMENTAL PROCEDURE

Meat was available from steers of known history. These steers were produced at Bluebonnet Farm, Texas Agricultural Experiment Station Substation No. 23. Studies emphasizing the effect of certain factors in production history and carcass quality on palatability will be published elsewhere. Twenty-four steers were available in 1953 and thirty-eight in 1954.

Three-quarter inch steaks were obtained from the short loin and bottom round. The bone was removed from the loin steak to facilitate freezer storage and to help prevent cupping during broiling. The individual steaks were wrapped, frozen at –20°F. and stored at 0°F. until the tests could be made. In 1954, steaks 3 and 4 on each side from each cut were used for palatability tests and steaks 1, 2, 5, and 6 for chemical analyses. The steaks which were ground for the raw samples were: loin, 1 and 6 left, 2 and 5 right; bottom round, 1 and 5 left, 2 and 6 right. Steaks used for the cooked samples were: broiled loin, 1 and 6 right, 2 and 5 left; broiled bottom round, 1 right and 6 left; and braised bottom round, 2 left and 5 right.

Cooking conditions. The steaks were cooked well-done. Broiling and braising methods have been described in detail by Cover and Shrode *(5)*. The broiler was preheated to 200°C. (392°F.). The steak was browned well on one side (approximately 25 minutes), turned, and browned on the other side (approximately 10 minutes). Use of steak

thermometers was not successful in ¾-inch steaks. In 1953, the broiled bottom round steaks were sometimes slightly pink instead of gray. In 1954, this undesirable effect was overcome by using a table of cooking times based on weight. Perhaps the reason it was successful was that the heavier steaks in each cut were also longer and wider while similar in thickness. The braising time after browning was standardized at 45 minutes in steam. The steak temperature reached 100°C. in about 20 minutes and was held there for about 25 minutes *(5)*.

Sampling for palatability and shear tests. Paired cuts from the right and left sides of the same animal were cooked—one by broiling and its pair mate by braising. Use of two pairs of cuts permitted a steak from the left and right sides of the animal to be broiled and a right and left to be braised. The entire steak was cooked, but after cooking an individual muscle was used for scoring and shearing tests. The muscles used were: *longissimus dorsi* in loin and *biceps femoris* in bottom round.

Shear force values were obtained with a Warner-Bratzler shearing device. Four cores ½-inch in diameter were obtained, two at one end of the steak and two at the other. The cores were so thin that only one shear from each core was obtained, making a total of 4 shears from each steak.

Palatability was scored by a panel of 5 judges. Composition of the judging committee was uniform within the tests for one year, but two judges serving in 1953 were replaced for the 1954 tests. The samples used were narrow strips of meat—not an entire steak—taken from the area in the central portion of the steak and between the two sets of cores. Each sample from a broiled steak was paired with a sample from a similar position in the braised steak of the pair. The judges used a score card of adjectives weighted from 0 to 10 wherein: 10 is *excellent;* 9, *very good;* 7, *good;* 5, *medium;* 3, *poor;* 1, *very poor;* and 0, *unacceptable*. The meat was scored for juiciness, flavor of lean, and tenderness. Steaks were tested for palatability from 10 of the 24 animals in 1953 and from all 38 in 1954.

Palatability scores have the serious disadvantage that the standards of the judges may not remain the same throughout the test, particularly if sampling periods are widely separated. To prevent bias among the variables in the present study, 8 samples were tested at each judging session. They were broiled and braised loin, and broiled and braised bottom round from each of two animals. At another session on the same day or the next day, the second pair of steaks from each cut from the same animals was tested so that an average of a left and right cut was possible. Thus, comparisons would be valid within each year for: the two methods of cooking within a cut; the two cuts by the same method of cooking; and the difference in response by the two cuts to the two methods of cooking.

Chemical analyses. Analyses for moisture and ether extract were made on the raw meat of *longissimus dorsi* in loin and of *biceps femoris* in bottom round. The analytical methods were essentially those described in AOAC *(1)*.

Total nitrogen and collagen nitrogen were determined on raw meat from 18 of the 24 steers in 1953 and from 26 of 38 in 1954 and on cooked meat from 13 of the 1954 steers. The method was essentially that of Lowery, Gilligan, and Katersky *(15)* except that instead of determining collagen by their gravimetric procedure, the determination was made for collagen nitrogen by the Kjeldahl procedure in the filtrate after autoclaving. Retentions of collagen nitrogen were calculated as follows: total content in cooked meat was multiplied by 100 and divided by the total content in raw meat. Weights of the cut before and after cooking and the percentage content of raw and cooked meat were used in calculating the total content.

All chemical analyses were made on the meat after the outside edge of the muscle was trimmed away so that adhering fat and connective tissue would not influence the results. Where analyses were made on cooked meat the trimming was done before cooking so that only the weights after trimming were used in the calculations. Thus, collagen content in this study corresponds more closely with tenderness scores and shear force values than it would if the heavy connective tissue covering the muscle had been included.

RESULTS AND DISCUSSION

Average weights of the raw steaks used for palatability tests are given in Table 1. Steaks cooked for palatability testing were not weighed after

TABLE 1

Raw weight of steaks, judges' scores and shear values

Cut and muscle[1]	Method of cooking	Number of steaks averaged	Weight of raw steaks grams	Juiciness scores	Tenderness scores	Shear force values pounds
1953 tests						
Loin	Broiled	20	165	6.3	7.8	8.9
(l. dorsi)	Braised	20	164	5.4	8.6	7.6
Bottom round	Broiled	20	324[2]	6.7	6.3	7.9
(b. femoris)	Braised	20	332[2]	5.2	8.3	6.2
1954 tests						
Loin	Broiled	76	164	6.5	6.5	9.9
(l. dorsi)	Braised	76	162	5.2	6.7	9.9
Bottom round	Broiled	76	208[2]	7.8	6.9	8.7
(b. femoris)	Braised	76	208[2]	5.5	8.0	5.9

[1] The entire piece of meat was cooked but only the portion inside the muscle sheath was scored or sheared.

[2] In 1953 parts of adhering muscles (especially the *semitendenosus*) were always present in bottom round. In 1954 all other muscles were cut away and only *biceps femoris* was cooked.

cooking but those cooked for the chemical analyses were. Weight losses during cooking for broiled loin, broiled bottom round, and braised bottom round averaged 42, 41, and 44%, respectively. Hence, there was little if any difference in weight loss among these steaks. Data on ¾-inch steaks from other animals cooked as were these showed weight losses between 40 and 45% also. Although these weight losses seem high in comparison with 21% to 37% as reported by Griswold *(7)* and 33% to 34% reported by Clark, Wilmeth, Harrison, and Vail *(2)*, it must be remembered that the cooking conditions probably were more drastic than theirs but no more so than those used by many homemakers.

Juiciness scores indicated that the broiled steaks were juicier than the braised in both cuts and in both years (Table 1). The difference in tenderness produced by the two methods of cooking was not only smaller in loin steaks than in bottom round, it was smallest in the 1954 tests where the greater number of animals was involved and the broiling conditions were better standardized (Table 1). Analyses of variance of the 1954 data (upper part of Table 2) indicate that *biceps femoris* was made more tender by braising than broiling but that *longissimus dorsi* was no more tender braised than broiled. In another analysis of variance of these data "cuts within methods of cooking" were compared (Table 2). After broiling, the difference between the two cuts was not significant for tenderness scores but was significant at the 1% level for shears. This was the only case in which the results from scores and shears did not agree closely. After braising, the difference in tenderness between the two cuts was significant at the 0.1% level for both scores and shears. Thus the greatest differences in tenderness occurred in braising and in bottom round. Cover and Shrode *(5)* using only 4 animals found a greater difference between broiled and braised bottom round than between broiled and braised loin steaks. Their braised bottom round steaks were the most tender.

TABLE 2

Outline of analysis of variance of scores and shears for 1954 steers

Source of variation	Degrees of freedom	Mean square[1]		
		Juiciness scores	Tenderness scores	Shear force values
Analyzed by cuts				
Cuts	1	51***	46***	538***
Methods within cuts	2	138***	24**	149***
Loin	(1)	(69***)	(2)	(0.–)
Bottom round	(1)	(207***)	(46***)	(298***)
Animals within cuts and methods	148	1***	3***	6***
Samples	152	0.4	0.4	1
Total	303			
Analyzed by methods				
Methods	1	258***	33**	146***
Cuts within methods	2	35***	30***	345***
Broil	(1)	(65***)	(4)	(59**)
Braise	(1)	(4*)	(56***)	(631***)
Animals within cuts and methods	148	1***	3***	6***
Samples	152	0.4	0.4	1
Total	303			

* Significant at 5% level.
** Significant at 1% level.
*** Significant at 0.1% level.
[1] Error term for animals was samples. Error term for other sources was animals.

Correlation coefficients between tenderness scores and shear force values were calculated for the 1954 data. The coefficients for the 38 animals were: broiled loin—.839; braised loin—.891; broiled bottom round—.730; and braised bottom round—.863. All were not only highly significant but were high enough so that the shears accounted for 50 to 80% of the variability in scores. Ramsbottom and Strandine *(18)* obtained tenderness scores and shear force values for 50 muscles cooked to an internal temperature of 170°F. (76.7°C.) in lard maintained at 250°F. (121°C.). Correlation between their scores and shears was reported as 0.9. It may be noted that, in the correlations reported here, agreement between scores and shears appeared to be somewhat lower in broiled bottom round than in the others even though the four samples were presented on the same plate and the order of the samples on the plate was varied. While this coefficient did not differ significantly from the other three, it is suggested that the tenderness sensations available to the judges from tooth and jaw pressure (which should correlate closely with shears) may be modified by additional and contradictory sensations from tongue and cheek. The possibility that these additional contradictory sensations may be associated somewhat more closely with certain muscles cooked in certain ways should not be overlooked.

Methods for determining collagen nitrogen are still in the developmental stage. The greatest difficulty lies in extracting the muscle proteins from the connective tissue proteins. Muscle proteins are more easily extracted

from raw meat than from cooked meat because after cooking they have been coagulated and made less soluble. However, results for duplicate samples checked within 10% of the lower value and ¾ of them within 5%.

Work with the 1953 steers was done on raw meat only, using the *psoas major* muscle in loin, which appears to contain little coarse connective tissue, and the *biceps femoris* in bottom round, which has a rather heavy net of connective tissue. The collagen in raw *psoas major* and in raw *biceps femoris*, respectively, was 0.83 and 2.75 g. of collagen nitrogen per 100 g. total nitrogen. The tender muscle, *psoas major*, thus contained much less collagen nitrogen than the less-tender muscle, *biceps femoris*.

In 1954 the muscles used were *longissimus dorsi* in loin as the tender muscle and *biceps femoris* in the bottom round as the less-tender muscle. Raw *longissimus dorsi* in loin contained less collagen nitrogen than raw *biceps femoris* (Table 3).

TABLE 3

Content and retention of collagen nitrogen in 1954 steers

Basis of comparison	Number of animals averaged	Content of collagen nitrogen				Retention of collagen nitrogen[2]	
		Moist basis		Per 100 grams total nitrogen			
		L. dorsi	*Biceps femoris*	*L. dorsi*	*Biceps femoris*	*L. dorsi*	*Biceps femoris*
		%	%	grams	grams	%	%
Raw meat	26	0.067	0.105	1.88	3.04	....	....
	13[1]	0.072[1]	0.109[1]	2.01[1]	3.16[1]	....	....
Cooked meat							
Broiled	13	0.064	0.106	1.06	1.87	51	57
Braised	13	.....	0.065	.....	1.16	....	34

[1] Values of raw meat to correspond with those for cooked meat given below.

[2] Retention of collagen nitrogen was calculated according to the following formula:

$$\frac{\text{Wt. in g. of meat after cooking} \times \text{content per g. of cooked meat (moist basis)}}{\text{Wt. in g. of meat before cooking} \times \text{content per g. of cooked meat (moist basis)}} \times 100 = \% \text{ retention.}$$

Collagen nitrogen was converted into collagen by using 18.6% as the nitrogen content of collagen *(9)* so that comparisons could be made with the values reported by other workers. The collagen content on the moist basis averaged 0.36% for *longissimus dorsi* and 0.57% for *biceps femoris* in the raw meat of the 26 animals. Griswold, using the Lowery procedure, reported collagen values of 0.87%, 1.11%, and 1.17% for *semitendinosus* muscle in bottom round *(9)* and a range of 0.57% to 1.13% for three muscles in two or more pairs of rounds *(8)*. In none of her samples was the outside covering of the muscle removed. This would tend to give higher values than in the present study where the connective tissue covering of the muscle was removed. Wilson, Bray, and Phillips *(19)* used a modified Lowery method and reported the average collagen content of *longissimus dorsi* on the moist basis as 0.416%. Their samples were obtained over the 12th and 13th ribs—somewhat anterior to those in the present study—and from steers which graded from U. S. Commercial to U. S. Prime. Whether or not their determinations were made on trimmed muscles was not stated. They also reported the collagen content on the dry fat-free basis as 1.89%. It was 1.56% in the present study.

The difference in content of collagen nitrogen in the raw meat appeared to have been greater between *psoas major* and *biceps femoris* in the 1953 steers than between *longissimus dorsi* and *biceps femoris* in the 1954 steers. Thus the trend in content of collagen nitrogen appears to be in the direction expected *(3)*. Other workers also have reported on the comparative tenderness and comparative collagen content of these muscles. *Psoas major* has been reported to be more tender than *longissimus dorsi (11, 18)* and to have fewer collagenous fibers *(10, 18)*. But the reports in the literature on the relative tenderness and collagen content of *longissimus dorsi* and *biceps femoris* are not in as good agreement. *Longissimus dorsi* was found in one study to be more tender than *biceps femoris (11)* but only slightly so in another *(18)*. Histological ratings were the same for the two muscles in one study *(18)* but in the other study the statement was made that those muscles which are used more extensively by the animal had larger amounts of collagenous fibers, whereas, those with less activity had smaller amounts *(12)*. Leg muscles, such as *biceps femoris*, are supposed to be used more extensively than the rib and loin muscles, such as *longissimus dorsi*.

Rather large differences in collagen content of the same muscle from different animals were noted in the raw meat of the present study and may prove to be important. Some animals had irregular strands of heavy connective tissue even in *longissimus dorsi*. Sometimes these strands contained visible deposits of fat—sometimes not. No systematic way of recording was devised until after these tests were completed. But it seems likely that animals in which this condition was marked would have a higher collagen content than those in which it was less marked or was absent. The effect on tenderness after cooking can not be predicted. The cause of this condition is unknown. Wilson, Bray, and Phillips *(19)* also reported considerable variation in the percentage collagen between animals of the same grade and age. They used a modification of the Lowery method for collagen on the raw *longissimus dorsi* of 88 animals differing in age, grade, and sex. Harrison *et al. (10)* reported that the relative collagen content in certain muscles, as determined histologically, was higher in two of their four animals than in the other two.

In cooked meat, content and retention of collagen nitrogen in *biceps femoris* of bottom round were smaller in braised than in broiled steaks (Table 3). The difference in collagen nitrogen remaining in *biceps femoris* after broiling and braising was significant at the 1% level (Table 4). These data lend support to the theory that moist heat as used in this study was more effective in degrading collagen than the dry heat method, at least within the *biceps femoris* muscle. *Biceps femoris* also was more tender braised than broiled (Tables 1, 2). For comparison of collagen content with tenderness in the same animals, the average tenderness scores for *biceps femoris* in these 13 animals were 7.1 and 8.3 after broiling and braising, respectively, and the average shears were 9.2 and 5.4, respectively. Thus, these data lend support to the theory that tenderness is influenced by the actual amounts of collagen left in the meat after cooking, as has been assumed *(3)*. Winegarden, Lowe, Kastelic, Kline, Plagge, and Shearer *(20)* reported the shearing strength of strips from the external portion of the aponeurotic sheet. These values indicated marked tendering

TABLE 4

Outline of analyses of variance of collagen nitrogen content of cooked meat for 13 of the 1954 steers

Source of variation	Degrees of freedom	Mean square[1]
One cut cooked by two methods		
Methods of cooking bottom round	1	21,730**
Animals within methods of cooking	24	1,817***
Samples	26	9
Total	51	
Two cuts cooked by one method		
Cuts after broiling	1	22,975***
Animals within cuts	24	1,201***
Samples	26	10
Total	51	

** Significant at 1% level.
*** Significant at 0.1% level.
[1] Error terms for animals was samples. Error terms for other sources was animals.

within 4 minutes at 95°C. but a much slower rate of tendering for the next 60 minutes at this temperature. Whether a temperature of 100°C., as was used in braised steaks, would have followed the same pattern is not known. The meat temperature in braised steaks was above 95°C. longer than 4 but less than 60 minutes. It is not possible to determine whether the collagen in these steaks did or did not respond to heat in the same way as did the aponeurotic sheet. However, the following comparison may be noted. It appears from the figure in which the data of Winegarden *et al.* were presented, that the drop in shear force was from about 25 to less than 10 pounds, a loss of somewhat more than 60% in shearing strength in 25 minutes at 95°C. Loss of collagen nitrogen in bottom round steaks in the present study was about 65% during braising where steak temperatures were at 100°C. for about 25 minutes.

Content and retention of collagen nitrogen in *longissimus dorsi* of loin could be secured for only one method of cooking because of the limited amount of meat available. Therefore comparisons for methods of cooking within *longissimus dorsi* can not be made.

Comparison of the two muscles cooked by broiling indicate that retentions of collagen nitrogen did not differ greatly (Table 3). However, it must be noted that the actual content of the two muscles was different and that after broiling less collagen was found in broiled loin steaks than in broiled bottom round steaks. The difference in collagen nitrogen remaining in the two muscles after broiling was significant at the 0.1% level (Table 4). If tenderness is influenced by the actual amounts of collagen left in the meat after cooking, as has been assumed *(3)*, this might lead one to expect that broiled loin steaks with less collagen would be more tender than the broiled bottom round steaks. Neither the scores nor the shears of the 1954 data (Table 1) lend support to this conclusion. The averages in these 13 animals for tenderness scores in broiled loin and broiled bottom round were 7.2 and 7.1, respectively, and for shears were 9.1 and 9.2, respectively, whereas the grams of collagen nitrogen per 100 g. of total nitrogen were 1.06 and 1.87, respectively. These data indicate that actual collagen content inside the muscle after broiling may not be a major factor

in determining relative tenderness of muscles from different positions in the carcass when steaks are broiled well-done.

Kastelic *(13)* suggested that the sodium hydroxide–soluble fraction which is discarded in the Lowery method may contain an integral component of ground substance, the very matrix of connective tissue. Since connective tissue is thought to be composed of amorphous as well as fibrillar components, Kastelic thought that the role of connective tissue in the tenderness of meats could not be determined until the role of each of these multiple components is known. The results in the present study with moist and dry heat methods of cooking indicate that collagen content was associated with tenderness when the same muscle *(biceps femoris)* was cooked by different methods. But when the tenderness of the two muscles was compared by the same method of cooking (broiling) collagen content was not associated with tenderness. How greatly elastin or ground substance or even the muscle proteins were involved in these tenderness comparisons is not known.

Other observations in regard to the effect of cooking on these two muscles were made in 1954. After braising, the muscle fibers of these two cuts differ in a rather remarkable way—at least in certain animals. During carving for the judging tests, small bundles of muscle fibers in braised *longissimus dorsi* sometimes fell apart readily into strings. But these strings were tough, indicating that these bundles of fibers were not easily broken across the grain. This is the familiar condition in which the connective tissue appears softened but the muscle proteins, toughened. In contrast to this is the condition in braised bottom round steaks. After braising, bundles of muscle fibers in *biceps femoris* in the same animals not only separated easily but also broke readily across the grain. This gave these braised bottom round steaks a mealy feel in the mouth. This mealy feel in the mouth was associated with dryness and unusual tenderness. Similar conditions have been reported previously by Cover *(4, 6)* in 3½-inch roasts cooked well-done at extremely low oven temperatures (80°C. and 90°C.) and by Griswold *(7)* in 1½-inch roasts cooked well-done at a constant low oven temperature of 121°C. (250°F.).

Perhaps this observation may be related to individual muscle fibers. The usual conception of muscle fibers has been that they are short, are held together end to end, and that many short fibers are meshed all along the length of the muscle. However, Lowe *(14)* and Paul *(16)* reported that in their histological studies with meat they have seldom if ever seen such an arrangement of the ends of muscle fibers. They believe that each individual muscle fiber extends the entire length of the grain of the muscle and that connective tissue at each end connects it with the tendon instead of to other muscles in tandem. If this is true, a muscle which breaks readily across the grain also breaks readily across the long axis of the muscle fiber. Whether the friableness of the muscle fibers in braised *biceps femoris* was caused by degradation of the collagen in the sarcolemma or was caused by changes in the muscle proteins is not known. Histological studies of Paul, Lowe, and McClurg *(17)* and of Harrison *et al. (10)* have shown that during aging disintegration of the protoplasm within the sarcolemma occurred and at times the sarcolemma was broken also. Because tenderness

increased with aging, as tested by both scores and shears, it may be inferred that these protoplasmic changes were involved in the increase in tenderness. The possibility that similar disintegration may be brought about by conditions other than aging should not be overlooked.

In some animals, at least, the muscle fibers of *longissimus dorsi* and *biceps femoris* apparently react alike to broiling, but not to braising. This difference in muscle fiber response of these two cuts to braising may be a factor of great importance in the tenderness of cooked meat.

CONCLUSIONS

Well-done steaks from loin and bottom round were more juicy broiled than braised. Steaks from the *biceps femoris* muscle in bottom round were more tender braised than broiled. Steaks from *longissimus dorsi* in loin were little if any different in tenderness when cooked by the two methods. Highly significant correlations were obtained between tenderness scores and shear force values by both methods of cooking and for both cuts.

Collagen nitrogen content in raw meat was found to be lowest in *psoas major* and highest in *biceps femoris*. The value for *longissimus dorsi* in loin lay between these two extremes. In cooked *biceps femoris*, content and retention of collagen nitrogen were smaller in braised steaks than in broiled, indicating perhaps that the moist heat method was more effective in degrading the collagen in *biceps femoris* than was the dry heat method. In cooking *longissimus dorsi*, content and retention of collagen nitrogen were obtained for broiled steaks only. The steaks from loin and bottom round after broiling had similar retentions of collagen nitrogen but the broiled loin steaks were significantly lower in actual content of collagen nitrogen.

The results with moist and dry heat methods of cooking indicate that collagen content was associated with tenderness when the same muscle *(biceps femoris)* was cooked by different methods, but when the tenderness of the two muscles was compared by the same method of cooking (broiling) collagen content was not associated with tenderness.

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THERMAL DESTRUCTION OF PEROXIDASE IN VEGETABLES AT HIGH TEMPERATURES[a]

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In developing methods for improving the quality of canned foods, high temperature short-time and aseptic canning techniques are assuming greater importance as means of achieving these ends. Recently Guyer and Holmquist *(3)* have shown that in the application of high temperature short-time sterilization procedures for low acid foods the regeneration of peroxidase enzyme activity is a factor that must be considered. They showed that under such conditions peroxidase enzyme regeneration does occur in canned peas and that the time-temperature relationship for high temperature processes must be clearly established for complete enzyme destruction in some products to insure flavor stability. The regeneration of peroxidase was associated with the development of off-flavor in canned peas. It was pointed out that in cases where the enzymes regeneration problem exists, longer heating times must be used to meet enzyme destruction requirements.

The phenomenon of peroxidase regeneration has been recognized and discussed by Schwimmer *(9)*, Balls *(1)*, and Kaplan, Esselen, and Fellers *(4)*. Esselen *(2)*, in discussing the thermal inactivation of enzymes as related to heat-processed canned food products, stated that it has been frequently assumed that the heat treatment given canned products to destroy microorganisms capable of causing bacterial spoilage was more than adequate to destroy enzymes. While this assumption is undoubtedly true in low-acid foods that require a relatively long process to destroy bacterial spores, it may not necessarily hold in acid products such as fruits and pickles, which are usually given a relatively short and mild heat treatment. The thermal destruction of peroxidase in pasteurized fresh pack pickles has been studied extensively at this laboratory, *(5, 6, 7)*. Peroxidase activity was associated with the development of off and stale flavors in this product.

The present investigation was carried out as a survey to observe the comparative heat resistance of peroxidase in a number of different fresh vegetables. Data were obtained on the degree of heating required to prevent regeneration as well as the time and temperature relationships needed to destroy enzyme activity when the substrate was tested immediately after being heated.

EXPERIMENTAL PROCEDURE

Preparation of samples. Approximately 200-g. samples of cut raw vegetable were mixed 2:1 or 1:1 parts by weight with distilled water and were blended for 5 minutes in a Waring blender. The mass was then strained through 4 layers of cheese cloth. The

[a] Contribution No. *1019* of the University of Massachusetts College of Agriculture Experiment Station.

filtrate, containing small particles of plant tissue, was used as the test substrate. It was held at 35°F. prior to use (the holding periods did not exceed 48 hours).

Heating samples. Samples were heated in steam at different times and temperatures in a thermoresistometer as described by Pflug and Esselen *(8)*. Essentially the same techniques were followed as in the determination of thermal death times of bacterial spores with the exception that aluminum rather than tin plate sample cups were employed. A disc of thin rapid filter paper was placed in the bottom of each cup to prevent the sample from forming a droplet. Samples of 0.01 ml. of the vegetable purée were placed into the thermoresistometer cups by means of a 0.2-ml. serological pipette graduated in hundredths of a ml. When the sample cups were removed from the thermoresistometer at the end of the heating period, they were dropped into clean test tubes rather than in tubes of culture media as in bacteriological thermal death-time tests. Six sample cups were heated for each time and temperature interval. When the cups were removed from the machine, they were placed in groups of six in petri plates.

Determination of peroxidase activity. The peroxidase activity of each individual sample was determined directly in the thermoresistometer cups by a "micro" modification of the o-phenylenediamine hydrochloride method. To each cup were added 0.1 ml. distilled water, 0.05 ml. phosphate-citrate buffer (pH 5.1), and 0.02 ml. of 0.3% hydrogen peroxide. The cups were then agitated to mix the reagents and were allowed to stand for 5 minutes. At the end of this time 0.02 ml. of a 1.0% aqueous solution of o-phenylenediamine hydrochloride (Opda) were added. (This solution is unstable and should be made up fresh every 4 hours. After 5 minutes 0.1 ml. of saturated sodium bisulfite solution was added to stop the enzyme reaction. Peroxidase activity produces an orange-red color. The presence and degree or the absence of peroxidase enzyme activity was evaluated visually and compared with control samples that had been heated sufficiently to assure the destruction of enzyme activity.

Peroxidase regeneration. To test for the regeneration of peroxidase activity replicate groups of heated samples were held at 35°F. for 2 and 7 days prior to testing for the presence of this enzyme.

Tests conducted. Depending upon the heat resistance of peroxidase in a given vegetable extract, thermal resistance tests were made at one to eight different temperatures throughout the range of 215° to 300°F. Seventeen different vegetables were investigated including 6 different lots of green beans, 4 lots of yellow turnips, and 3 lots of broccoli.

Treatment of data. The survival and destruction endpoints were plotted on semi-logarithmic paper, and thermal destruction curves were plotted in the conventional manner. From these curves, F_{212}, F_{250}, and F_{290} values (time for destruction at 212°, 250°, and 290°F., respectively) and z values (slope of thermal destructive curve, expressed as number of degrees Fahrenheit required for the curve to traverse one log cycle), were derived. F_{212} values were obtained from an extension of the curve from 215°F. to 212°F.

RESULTS AND DISCUSSION

The results are summarized in Table 1. Where F_{212} data only are presented, the peroxidase in the vegetable indicated had a relatively low degree of heat resistance and not enough points could be determined in the working temperature range of the thermoresistometer to establish a destruction curve and z value. It may be seen that in the temperature range of 215° to 300°F. the peroxidase in vegetables had generally high z values as compared to the z values of 18° frequently assumed for bacterial spores. These observations are in agreement with those of Guyer and Holmquist *(3)* who found evidence that peroxidase in peas had a relatively high z value in this temperature range. Rather marked differences in the thermal resistance characteristics of peroxidase in different samples of a given vegetable are evident, as indicated in Table 1.

Significant evidence of peroxidase regeneration was obtained in green beans, broccoli, and yellow turnips (extensive regeneration tests were not

TABLE 1

Thermal resistance characteristics of peroxidase in vegetables

Vegetable	Inactivation after heating				To prevent regeneration			
	F_{212}	F_{250}	F_{290}	z	F_{212}	F_{250}	F_{290}	z
Artichoke, Globe	.080	.....	.....	.....	.....	.....	.....	.....
Beans, green	1.700	0.570	0.180	80.0	6.50	1.05	0.154	48.0
Beans, green	.....	.....	.....	.....	8.90	2.30	0.540	64.1
Beans, green	1.800	0.510	0.134	69.1	.....	3.70	0.460	44.4
Beans, green	.....	.....	.....	.....	.....	1.95	0.050	25.7
Beans, green	.....	.....	.....		.....	3.30	0.060	23.0
Beans, green	1.200	0.460	0.020	43.9	2.90	0.70	0.165	64.8
Beets	.007	.....	.....	.....	.....	.....	.....	.....
Broccoli	5.500	1.400	0.320	63.0	13.80	1.90	0.240	44.5
Broccoli	.....	.....	.....	.....	9.45	2.98	0.870	75.3
Broccoli	22.000	3.900	0.620	50.8	117.00	11.00	0.910	37.2
Cabbage	0.397	.....	.....	.....	.....	.....	.....	.....
Carrots	0.006	.....	.....	.....	.....	.....	.....	.....
Cauliflower	0.700	.....	.....	.....	.....	.....	.....	.....
Cucumber	2.600	.....	.....	11.3	.....	.....	.....	.....
Parsnip	0.010	.....	.....	.....	.....	.....	.....	.....
Peppers, green	1.300	.....	.....	9.4	.....	.....	.....	.....
Potato, white	0.500	0.004	.....	18.5	.....	.....	.....	.....
Radish	0.155	.....	.....	.....	.....	.....	.....	.....
Spinach	0.050	.....	.....	.....	.....	.....	.....	.....
Squash, Hubbard	2.000	.....	.....	8.2	.....	.....	.....	.....
Squash, Summer	0.444	.....	.....	.....	.....	.....	.....	.....
Turnip, white	9.800	2.100	0.420	57.5	.....	.....	.....	.....
Turnip, yellow	10.300	3.100	0.870	72.5	.....	.....	.....	.....
Turnip, yellow	16.000	4.000	0.960	64.0	28.50	6.70	1.500	60.5
Turnip, yellow	.....	.....	.....	.....	26.20	6.20	1.330	60.5
Turnip, yellow	.....	.....	.....	.....	81.00	14.50	2.390	51.1

made with the other vegetables studied). It is obvious that the degree of heating required to prevent regeneration of the enzyme during subsequent storage for a week at 35°F. is considerably greater than that required to temporarily inactivate it, as indicated by tests made immediately after the samples were heated. These observations, confirming those of Guyer and Holmquist *(3)*, indicate that in the high temperature short-time processing of canned foods particular attention should be paid to the inactivation of enzyme activity in addition to the destruction of bacterial spores.

SUMMARY

Data on the thermal destruction of peroxidase in seventeen different vegetables in the temperature range of 215° to 300°F. were obtained by means of a thermoresistometer. The degree of heat required to prevent regeneration of enzyme activity during subsequent storage was two to four times greater than that required to destroy the enzyme on a basis of tests made immediately after heating. The thermal resistance of peroxidase was characterized by much higher z values than are found with bacterial spores. The peroxidase of green beans, broccoli, and turnips exhibited marked heat resistance.

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THE RESPIRATORY ENZYMES OF MEAT. III. THE INFLUENCE OF VARIOUS IONS ON BEEF SUCCINOXIDASE

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Inorganic salts play a central role in meat processing. They are used in large quantity for their promotion of desirable flavoring, coloring, and binding. Since they are highly reactive chemicals, they may be expected to exert influences, both favorable and unfavorable, for which they were not intended. Moreover, commercial grades of inorganic salts carry as contaminants far from inconsiderable amounts of other inorganic salts.

The intention of the present study was to examine the influence of a number of ions on beef succinoxidase, an enzyme system which contributes to the oxygen tension in meat *(3)*. The literature is inconsistent regarding ionic effects on succinate oxidation. This partly reflects the variable choice of tissue and partly the difficulty in assigning an effect to a specific ion or the entire salt. Thus, both stimulatory and inhibitory effects have been reported for sodium chloride *(5, 8)* and for calcium *(4, 6)* and magnesium *(2, 6)* ions. In addition, an antagonism between magnesium and calcium ions has been reported *(7)*.

EXPERIMENTAL

Succinoxidase assays were performed manometrically. Beef muscle tissue, which had been frozen within 18 hours of slaughter, was thawed within 2-4 hours of use. It was ground in a hand grinder through ⅛ inch holes and patted with filter paper. 0.5 g. samples were weighed out and added to Warburg flasks. The only other components were sodium succinate, the additive under study, and water, with a total volume of 3.0 ml. All salts were reagent grade. In the experiments of Table 1, the succinate concentration was 0.07 M, and the assay temperature was 25°C. In the experiments cited subsequently, the succinate level was set at 0.03 M and the assay temperature at 37°C. In the absence of buffer, the initial pH was approximately 6.0 and changed negligibly during the course of the assay.

TABLE 1

The action of inorganic salts on beef muscle succinoxidase

Salt	Per cent inhibition at various concentrations				
	10^{-4}M	10^{-3}M	10^{-2}M	10^{-1}M	1 M
Sodium chloride	6	0	9	65	100
Sodium sulfate	4	4	10	30	74
Potassium chloride	1	0	6	40	91
Calcium chloride	13	11	49	98	100
Magnesium sulfate	....	12	29	65	79

RESULTS

Table 1 outlines the action of several salts on beef succinoxidase. These are net effects, superimposed on any influences of 0.07 M levels of sodium and succinate ions and the ions present in the unwashed, undialyzed tissue. It is observed that each of

the salts at 1.0 M is capable of blocking more than half the enzyme activity, and all except sodium sulfate are capable of this blockage at 0.1 M. Calcium chloride inhibits half the activity at 0.01 M. Comparison of the sodium sulfate, sodium chloride, and calcium chloride data suggests an inhibitory action by calcium ion, as well as an inhibitory action by chloride ion and/or a stimulatory action by sulfate ion.

In another experiment, a comparison was made between 0.01 M calcium chloride, 0.01 M calcium nitrate, and 0.02 M sodium nitrate in the presence of 0.03 M sodium succinate as substrate. The percentage inhibitions were, respectively, 56, 62, and 62. Several explanations are possible: (a) inhibitions by calcium and sodium ions; (b) inhibitions by chloride and nitrate ions; or (c) inhibitions by all four ions. The data do not permit a clear choice, but if calcium and chloride ions are both inhibitory, it appears, inferentially, that nitrate ion is, also.

Calcium ion appears to bind tightly one or more components of the beef succinoxidase system. Incubation of water-washed ground beef samples (2 g.) with 0.07 M sodium chloride, potassium chloride, calcium chloride, sodium sulfate, and sodium nitrate for 5 hours at 38°C., followed by filtration and thorough washing of the tissue with water, led to reduction of succinoxidase activity in only one case—calcium chloride treatment.

The mechanisms by which the other ions inhibit succinoxidase, while in contact with the enzyme system, are obscure. Consequently, the type of agent capable of interfering with the inhibition is not easily predictable. Three agents, sodium caprylate, serine, and glucose were tested for protection of succinoxidase against sodium chloride inhibition. Sodium caprylate, at concentrations of 0.002 M, 0.02 M, 0.2 M, and 1.0 M failed to influence the inhibition of 1 M sodium chloride. In the presence of serine, the inhibition by 0.2 M sodium chloride actually rose (from 21% to 25% with 0.05 M dl serine and 21% to 29% with 0.2 M dl serine). In the glucose study, the % inhibitions in the various systems were: 0.5 M sodium chloride, 53%; 0.5 M sodium chloride plus 0.5 M glucose, 34%; 0.5 M glucose, 34%. Apparently, glucose is capable of depressing the inhibition by sodium chloride, but it exerts an inhibition itself.

One of the relatively vigorous oxidative enzymes in meat is the cytochrome-linked α-glycerophosphate dehydrogenase *(3)*. This system is less sensitive than succinoxidase to inhibition by 1 M sodium chloride. For the former, Q_{O_2} was lowered from 2.8 to 1.7 (39%), and, for the latter, from 8.4 to 3.0 (64%).

DISCUSSION

The accelerating effects of sodium chloride and low oxygen pressure on metmyoglobin and methemoglobin formation were studied by Coleman *(1)*. No links between the two factors emerged. It is not unlikely that they are actually related, and one explanation is suggested by the present study. The optimum oxygen pressure for metmyoglobin formation is greater than the negligible pressure attending the formation of non-oxygenated, non-oxidized purple-red pigments. The environment for the latter is at least partly made possible by oxygen-consuming enzymes *(3)*. Any agent, such as an inorganic salt, which curtails these enzymes, would raise the oxygen pressure into the zone favoring met-pigment formation.

The partial inhibition of succinoxidase by sodium nitrate and sodium chloride may influence the course of meat curing. The maintenance of a highly reduced chemical environment is desirable, and an oxygen-consuming system such as succinoxidase would promote this state. By inhibiting the enzymes, the salts may be retarding the curing process.

In considering the applicability of data obtained by the method used here, it is well to note that salt concentrations at an inhibitory level are, relative to total amount of tissue, of the same order of magnitude as those added in commercial processing.

SUMMARY

Sodium chloride, sodium nitrate, sodium sulfate, potassium chloride, calcium chloride, and magnesium sulfate are capable of inhibiting beef succinoxidase. The oxidation of α-glycerophosphate is less sensitive to sodium chloride than the oxidation of succinate. The implications are discussed.

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MICROSCOPIC STRUCTURE OF APRICOT PUREES

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Apricot puree, as exemplified by the baby-food product, consist of tissue particles suspended in a solution of the soluble components of the fruit. The complexity of this structure varies according to the size and kinds of particles formed when the fruit is macerated by processing treatments. Increasing interest in the particulate structure of other fruit purees and juices has been indicated in several recent publications on the consistency of tomato products.

Tissue particles in tomato juices and concentrates have been described as "irregularly shaped masses often looking like cog wheels" and as forming an interlocking, fibrous structure *(3)*. Size and shape of these particles have been considered important to consistency *(5)*. Particle size distributions in tomato products also have been determined by mechanical sieving *(4)* and further elucidation of microscopic structure has demonstrated a likely relationship to gross viscosity *(1)*. Few descriptions of structure in fruit purees have been published, however, and there is only meager information on the sorts of tissue particles present. An exact definition of structure according to size and shape distributions of the particles is lacking and their relationship to consistency thus is not clear.

The extent to which distributions of particle size and shape may vary with processing seems a necessary basis for evaluating the role of particle structure in consistency. The present paper describes the results of preliminary studies designed for comparing the structure of apricot purees of different manufacture. Sampling methods and classification of the particles as to size, shape, and fruit tissue origins are presented.

METHODS

Purees of two different methods of manufacture were used. Although variety of fruit, its ripeness, and waste during manufacture are important in processing, they were not considered significant to the present objective. Of the purees, one was produced at this Laboratory and the other was commercially manufactured. In the Laboratory operation, whole, steam-blanched fruit was pitted and the flesh comminuted in a paddle-wheel pulper having an 0.027-inch sieve. The fruit received only this one sieving treatment. Whole, blanched fruit was put through a pulper with an 0.25-inch sieve in the commercial operation. The resulting coarse pulp, thus freed from pits, was pumped to a steam-injection chamber where it was flash heated to about 250°F. (120°C.) and forced through orifices of about 0.070-inch diameters. The heated pulp was further comminuted in a fine chopper and finished in a paddle-wheel finisher with an 0.020-inch sieve.

Microscopic observations and counts of individual tissue particles were made on samples of these purees diluted with 50 parts of water. This dilution was necessary for measurements and identification of clearly separated particles. One hundred grams of puree were thoroughly mixed with 5 liters of water and, before any settling could occur, a small portion was poured into a 100-ml. beaker. While this small aliquot was carefully stirred, a sample was removed with a large-opening medicine dropper, placed in a Sedgewick-Rafter counting chamber slide and covered with a coverglass. The chamber of this slide has a capacity of 1 ml. and is 1 mm. deep. The depth permits suspension of nearly all of the larger particles without shape distortion by the coverglass. An

additional advantage is that the known capacity of the chamber permits a quantitative estimation of the number of particles per unit volume of puree.

Three hundred or more particles were counted and classified in each of 3 or more slide-chamber preparations for each puree in order to minimize sampling error. Numerical distributions of the histological and shape classes of the particles then were calculated from a basis of 1000 or more counted particles and were expressed as percentages.

Measurements of individual particles were made with a calibrated eye-piece micrometer at 100 × magnification. Fifty to 100 particles of each class were measured in the order of their appearance under the micrometer when the slide was moved by the mechanical stage of the microscope. After one series of measurements across the slide was completed, the slide was moved a sufficient right angle distance to insure against repeated measurements during a return traverse.

The particles were classified on a basis of general characteristic shape for each class. This shape—spherical, cylindrical, or rectangular—was assumed to be typical for the class. Calculations of statistical volumes of the particles were made from the average, or arithmetic means of (a) diameters of spherical particles, (b) diameters and lengths, respectively, of cylindrical particles, and (c) thickness, width and length, respectively, of the rectangular particles. This calculated volume, when multiplied by the per cent numerical distribution of the class under consideration, gave a relative volume for that class. The space occupied in the two purees by the different particle classes then was compared in ratios or per cents of total relative volumes so obtained.

Expressions of volumetric distributions by these methods are arbitrary. The volume of a particle class, as obtained frm the arithmetic means of the dimensions of the particles, may not represent the true average particle volume, or average of the sum of individual particle volumes. Use of the volume of the arithmetic mean dimensions, however, provides a statistical method for calculating a relative value for a class of particles and has been used in studies of small particles of different shapes and sizes *(2)*. Replicate determinations by the described method agreed within ± 5 per cent and was found practical for demonstration of structural differences in these apricot purees.

OBSERVATIONS AND RESULTS

Particle classification. Microscopic examination revealed that most of the tissue particles consisted of intact, separated parenchyma cells in which the insoluble, granular matter of the contents was readily evident. A few cells were either crushed or reduced to cell wall fragments. Other particles consisted of clusters of unseparated parenchyma cells, fragments of vascular and skin tissues, and minute debris freed from broken cells.

The tissue regions represented by the particles could be identified. Figures 1 and 2, from sections of intact fruit, illustrate tissue regions most representative of the origins of the puree particles. Histological and shape classes of the puree particles were thus established:

(1) *Spherical cells.* These included all intact, separated parenchyma cells with length to width ratios less than 3:1 (Figs. 3 and 4). This ratio was found convenient because most of the fruit flesh is made up of parenchyma cells having axial ratios of less than 3:1. Although described here as spherical, most of these cells are polyhedral, about 14-sided, as is typical of plant parenchyma, and show 5 to 7 sides in sections of intact tissue (Figs. 1 and 2). Only about 10% of these cells showed shapes approaching a 3:1 axial ratio and even these had rounded ends. The spherical cells form the outer and middle fleshy portions of the fruit and vary greatly in size; the smaller are closely associated with the vascular bundles and skin tissues (Figs. 1 and 2).

(2) *Oblong cells.* These are intact, separated parenchyma cells having axial ratios of 3:1 and greater (Figs. 3 and 5) and were consequently considered to be cylindrical. They range from about 20 × 100 μ to 100 × 800 μ or more in dimensions and many of the more elongated cells have diameters of 60 μ or less. Most of the parenchyma tissue adjacent to the pit of the fruit is composed of oblong cells. Smaller oblong cells occur in the parenchyma bordering the vascular bundles, and some are closely associated with the skin.

This general distribution of parenchyma cell shapes has been described for other drupe fruits *(9, 10)*, but the inner and outer zones of elongated and spherical cells, respectively, appear to be more pronounced in raspberry drupelets *(8)* than in such fruits as apricots, cherries, and plums.

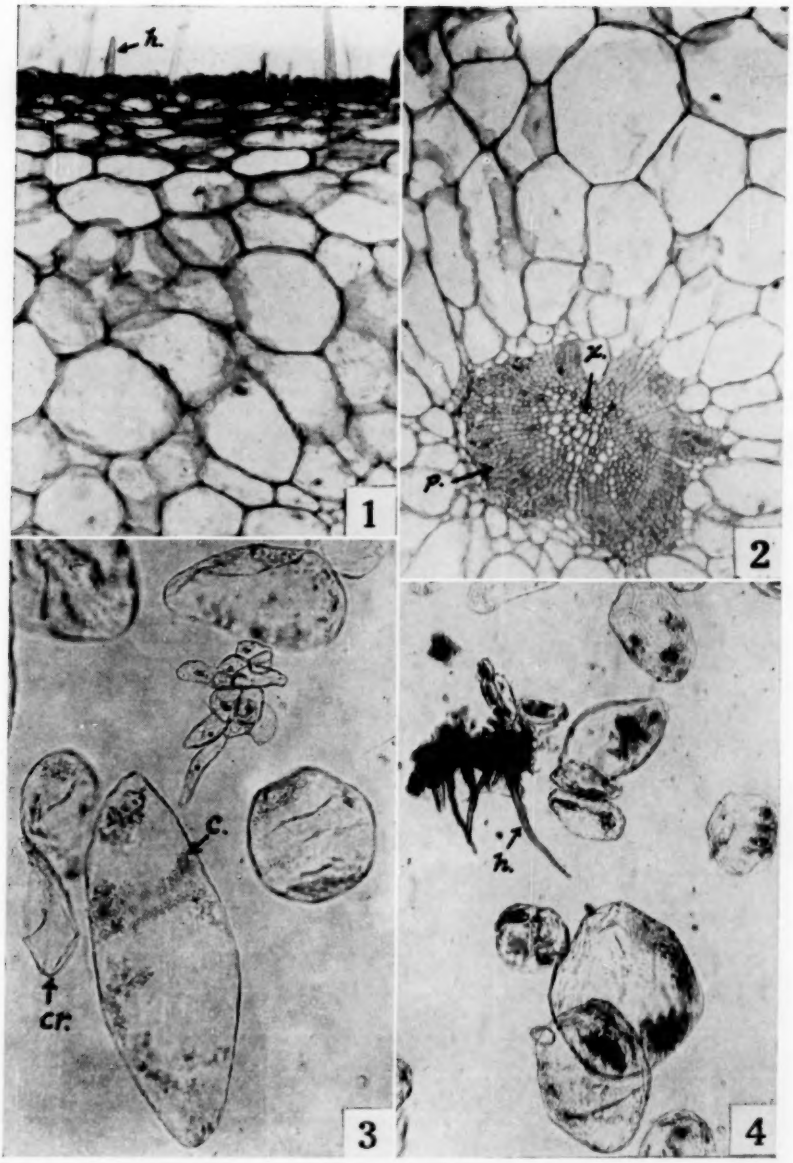

Figure Legends

Figures 1 and 2: Photomicrographs of stained sections (15 μ thick) of ripe apricot fruit, $\times$ 100. Fig. 1—skin and underlying tissues; note thick walls of epidermal and hypodermal cells, and epidermal hairs (h). Fig. 2—approximately the middle fleshy region of the fruit and showing a vascular bundle in transverse sectional view; x—xylem, p—phloem.

Figures 3 and 4: Photomicrographs of representative tissue particles in apricot puree, $\times$ 100. Fig. 3—separated, intact, parenchyma cells and small cell cluster; c—granular contents, cr—crushed cell. Fig. 4—separated, intact cells and skin tissue fragments; h—epidermal hairs.

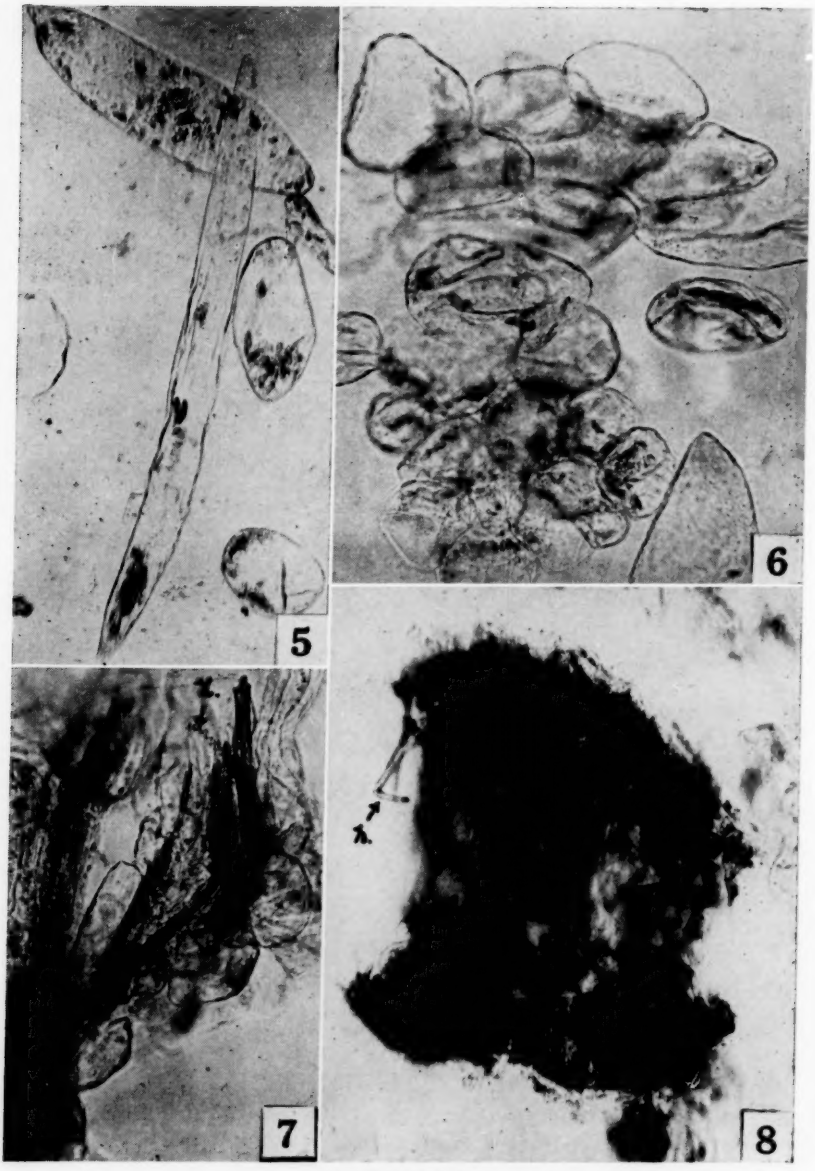

Figure Legends

Figures 5 to 8: Photomicrographs of representative tissue fragments in apricot puree, × 100. Fig. 5—separated, intact oblong and spherical parenchyma cells. Fig. 6—two large clusters of intact parenchyma cells. Fig. 7—part of a vascular tissue fragment branched at one end and showing spiral wall thickenings of a xylem element (x). Fig. 8 —large fragment of skin tissue showing epidermal hairs (h) on top surface.

(3) *Cell clusters.* These are composed of spherical and oblong parenchyma cells cohering in groups of 3 or more and are thus small chunks of the fruit flesh. Some clusters are tightly coherent while the cells of others are loosely coherent and are readily separated mechanically. These particles vary greatly in size (Figs. 3 and 6), depending on the degree of cell separation in a puree and also according to the tissue region represented by a cluster.

All clusters of intact parenchyma cells were arbitrarily considered as having cylindrical shapes. They tend to be oblong and it was found, for example, that the sum of individual cell volumes in a cluster, or $11.2 \times 10^5 \mu^3$, compared with the statistical volume of $12.9 \times 10^5 \mu^3$—calculated from the arithmetic means of the dimensions of the cells times the number of cells in the cluster. When the volume of the cluster was calculated as a cylinder, the value was $12.3 \times 10^5 \mu^3$. Apparently, there is fair agreement between calculated volumes no matter which method is used.

(4) *Vascular tissue fragments.* These consist of cohering cellular fragments or strands from the conducting bundle tissues of the fruit. In transverse section view, these bundles in the fruit present a circular outline (Fig. 2). The vascular fragments make up most of the stringy or fibrous "rag" in the puree. The ends of these fragments are sometimes branched (Fig. 7) and a number of the parenchyma cells usually remain attached. Smaller vascular particles often consisted of only a few xylem and phloem elements, the conducting tissues proper, while larger particles ranged between 2000 and 3000 μ in length and contained several hundred cells. All vascular fragments were considered to have cylindrical shapes.

The xylem elements are readily identified by their spiral or sometimes reticulate wall thickenings (x in Fig. 7). Both xylem and phloem cells, and immediately associated parenchyma cells are very narrow and elongated. These fragments consequently appear to contain more cell wall surface per unit volume and to possess a higher bulk density than do particles composed entirely of parenchyma cells.

(5) *Skin tissue fragments.* These fragments include the outermost surface cells (epidermis), immediately subjacent hypodermal cells, and varying amounts of underlying parenchyma cells. They are treated here as rectangular platelets with a thickness of about half of either width or length. The dimensions measured included the epidermal hairs because the hairs are closely enough spaced to increase the over-all particle volume (Figs. 4 and 8). Some skin fragments consist of only a few epidermal cells and a single hair; others exceed 1000 μ in greatest dimension. In general, these fragments may be classed as "rag" and are comparable to the vascular fragments in bulk density and large amount of cell wall surface.

Both the epidermal and hypodermal cells have thicker walls than do flesh parenchyma cells (Fig. 1). The walls of the hypodermal cells in particular stain strongly with cell wall stains and give strongly positive reactions to the histochemical test for esterified pectin *(6)*. These structural characteristics are common to the skin tissues of other pulpy fruits and it is generally accepted that these tissues in many fruits are rich in pectins.

(6) *Crushed cells and cell wall fragments.* Some cells become crushed and exhibit flattened, twisted shapes (*cr* in Fig. 3), but remain intact; others are torn open, or even reduced to cell wall fragments and their entire contents are freed into the serum. Particulate volumes were not calculated for this class although the particles were included in numerical distribution counts.

(7) *Stone cells.* Small clusters of tiny cells with extremely thick, pitted walls were sometimes observed. These were sclereids or stone cells, dislodged from the pits during pulping operations, which form the characteristic cellular structure of the pits of stone fruits *(8, 9)*. In these purees they amounted to less than 0.1 per cent of the total particles present.

(8) *Insoluble cell contents.* In addition to cellular particles, finely granular matter of the protoplasm and crystalloids freed from broken cells are suspended in the serum. These particles range in size from about 0.5 μ to dimensions below the resolving power of the microscope.

Particle size and distribution. The first six of these particle classes provide a basis for microscopic characterization. Each kind of particle may be recognized by clearly defined characteristics of structure and histological origin. Their distributions in a Laboratory and a commercial puree are compared in Table 1.

TABLE 1

Tissue-particle distribution in laboratory and commercial apricot purees.

Puree and particle class	Characteristics				
	Average dimensions (microns)	Volume of average particle ($\times 10^6$ μ^3)	Relative volume of class ($\times 10^6$ μ^3)	Per cent numerical distribution	Per cent of total relative volume
Laboratory:					
Spherical cells	147 ± 110	16.6	91.3	55	27.3
Oblong cells	75 × 385	15	33	22	10
Cell clusters	236 × 460	200	200	10	60
Vascular fragments	100 × 530	42	8.4	2	2.5
Skin fragments	115 × 230 × 315	8.3	0.83	1	0.2
Crushed cells and wall fragments	.....	.....	.....	10	....
Commercial:					
Spherical cells	140 ± 107	14.4	77.8	54	67.5
Oblong cells	70 × 340	13	24.7	19	21.9
Cell clusters	130 × 340	45	9	2	8.9
Vascular fragments	30 × 320	9	1.8	2	1.5
Skin fragments	30 × 60 × 160	2.9	0.29	1	0.2
Crushed cells and wall fragments	.....	.....	.....	22	....

Because size varies greatly between as well as within each particle class, numerical distributions alone may not reflect the actual difference between purees. Structural differences in these purees are more satisfactorily revealed by comparing their statistical particulate volumes. In Table 1, volumetric distributions are expressed as percentage of total relative volumes which were calculated as described from microscopic measurements.

Measurements of crushed cells and cell wall fragments were not included in Table 1. The average wall thickness of apricot parenchyma cells is less than 1 micron; the crushed cells and wall fragments, thus, represent tiny, irregularly shaped platelets which occupy a small fraction of the total particulate volume. Such particles may be important, however, when present in large enough numbers.

Differences in these two purees are shown in the numerical distributions of cell clusters and crushed cells (column 3, Table 1). A wide range of cell size, indicated by the deviation (± values) for spherical cells in column 1, is characteristic of the size variation which both spherical and oblong parenchyma cells exhibit in the intact fruit. Comparable variation in parenchyma cell size has been observed in apple flesh *(7)* and in other fruits *(8, 9, 10)*. An even greater range in particle size was observed for cell clusters and skin and vascular fragments.

These two purees also show marked differences in calculated particle volumes and volumetric distributions (columns 2 and 4, Table 1). Sixty per cent of the calculated statistical volume of the particles in the Laboratory puree consists of cell clusters as compared with 9% in the commercial puree. Ninety per cent of the calculated statistical volume in the commercial puree is composed of intact, separated cells as compared with less than 40% in the Laboratory puree. In addition, differences may be seen in the size of skin and vascular tissue fragments which are appreciably larger in the Laboratory puree than in the commercial product.

In general, these differences reflect the degree of cell separation which can be observed readily in different purees. Other structural distinctions in which fruit purees might differ are also revealed—as, for example, the amount of crushed and broken cells. Such variations may relate to manufacturing treatments or to other variables not presently considered.

DISCUSSION

Microscopic characterization of the suspended tissue particles aids in understanding the physical make-up of apricot puree. Statistical differences

in particle size and shape may be expressed volumetrically, as here, or in terms of particulate surface area. Thus, specific particle data are available for statistical analyses as in other commercial products in which small particle statistics have been studied *(2)*.

The individual particle characteristics are clearly revealed for microscopic measurements in the diluted puree. Numerically, about three-fourths of the particles in each puree described here are intact, separated flesh parenchyma cells and a majority of these are spherical to ovoid in shape. High density particles with irregular edges, such as skin and vascular tissue fragments, make up a small numerical percentage but the amount of cell wall matter which they represent may be significant.

This structural condition is different from the physical system of interlocking cogwheel particles described for tomato products and in which the "cog-like outer surface of the particles causes them to cling together by interlocking of the cogs" *(3)*. In some purees, as the commercial sample here, the intact, separated cells make up most of the particulate volume; in others, most of the particulate space may be occupied by irregularly shaped cell clusters. The edges of the clusters here, however, consisted mostly of rounded cell wall surfaces of the protruding, intact cells and in only a few instances were there observed torn border cells which formed ragged, cog-like surfaces. Other microscopic observations *(1)* have shown wide variation of structure in tomato products, ranging from juices containing many intact cells to those in which most of the cells were torn and ragged. Comparable differences in structure have been observed in tomato pastes at this Laboratory and have been experimentally produced in studies on apricot purees.

When apricot puree is examined in a thin, undiluted spread under a coverglass on a microslide, the tissue particles are crushed and appear to be tightly interlocked irrespective of their original shapes. Examination of thin, undiluted spreads through a stereoscopic microscope, without a coverglass, reveals a different situation. Vascular and skin tissue particles, together with the oblong parenchyma cells and cell clusters, appear to be stacked like log jams between the more spherical, separated cells. This sort of examination also grossly reveals the relative space and surface relationships of the different sorts of particles.

It is not unusual that many of the particles may be intact, separated cells or clusters of intact cells in a given puree. The middle lamellae of the cells of most ripe fruits are readily solubilized, or at least softened by cooking treatments such as blanching, and cell separation readily occurs during subsequent processing *(7, 11)*. The walls of parenchyma cells are characterized by their plasticity and microfibrillar porosity; they are not necessarily subject to breakage when the blanched fruit is put through a pulper as in puree manufacture. Mechanical abrasions and direct shearing forces, however, can rupture some cells—as exhibited by the two purees described here.

The sorts of tissue particles present in these purees are those expected to occur when the histological structure of the fruit is considered. Most of the edible fleshy portion of the fruit is composed of parenchyma cells and, although some skin and vascular tissues may be removed during siev-

ing, the gross distribution of tissues in the intact fruit is comparable with that found in the puree. Histological classification of the suspended tissue particles and their measurements thus provides a basis for characterizing fruit purees of different manufacture. It would also appear that this approach may be used in investigations on puree consistency.

SUMMARY

The microscopic structure of apricot purees consists of discrete classes of fruit tissue particles identifiable as intact, separated (1) spherical and (2) oblong parenchyma cells, (3) clusters of unseparated, intact parenchyma cells, fragments of (4) skin and (5) vascular tissues or "rag," and (6) crushed, broken cells and cell wall fragments. Differences in the numerical distributions of these classes of particles were found in two purees of different manufacture and appear to occur in other purees. Differences between these purees were also found in the average particle size in each class and in the volumetric distributions calculated from these averages. Clusters of unseparated cells and the intact, separated cells made up 60 and 37%, respectively, of the calculated particulate volume of a Laboratory-prepared puree. The volumetric distributions of these classes in a commercial puree were the opposite, being 9 and 90%, respectively. Numerical distributions of these particles, however, were essentially the same in each puree. Particulate characterization may be used to investigate changes caused by processing of fruit purees and pastes.

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EFFECTS OF FEEDING POLYOXYETHYLENE PREPARATIONS TO RATS AND HAMSTERS[a]

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Use of polyoxyethylene preparations in a wide variety of food products has aroused considerable interest in the effects which might follow their ingestion in food. On the basis of an evaluation of 48 published and unpublished reports available to it, the Food Protection Committee *(4)* of the National Research Council concluded in 1953 that the data "fail to demonstrate that polyoxyethylene stearates are safe for use in foods under all patterns of dietary consumption and for all segments of the population." Other publications have mentioned deleterious effects of polyoxyethylene and sorbitan compounds following oral *(13, 22, 29)* and parenteral *(3, 5, 14, 15, 19, 22, 23, 25, 27, 28)* administration to various experimental animals as well as after in vitro use *(1, 10, 12, 20, 21, 23)*.

Recently Krehl, Cowgill, and Whedon *(16)* reported that polyoxyethylene esters fed to rats and cats for varying periods of time did not have deleterious effects. Graham, Teed, and Grice *(7)* obtained negative results when they fed polyoxyethylene monostearate to rats in a bread diet for one year. Graham and Grice also reported negative results from feeding polyoxyethylene monostearate in a synthetic diet for 32 weeks *(6)*. The observations reported in the present paper involve the feeding of polyoxyethylene preparations[b] for a period of 21 weeks in rats and 39 weeks in hamsters.

PROCEDURE

Four experiments have been completed to determine the effects of moderate and high levels of several polyoxyethylene compounds in the diet. High levels were used in these experiments in conformity with recommended procedures for evaluation of the safety of chemicals in foods *(17, 18)*. In the first experiment, weanling male rats (Holtzman Rat Company, Madison, Wisconsin), distributed into 4 groups, were fed for 21 weeks on synthetic diets containing 25% levels of lard; cottonseed oil mono- and diglycerides (CMDG)[c]; polyoxyethylene monostearate (PMS No. 1)[d]; or polyoxyethylene sorbitan monolaurate (PSML)[e]. These diets differed only as to the control or test

[a] Data were presented originally in Exhibit 365, Ice Cream Hearings, Docket FDC 34A, 1952.

[b] These materials were offered on the open market for use in food products. They were mixtures of many chemical compounds and undoubtedly contained traces of unidentified impurities. The heterogeneous composition of polyoxyethylene stearates has been discussed at some length in a report issued by the Food Protection Committee *(4)*.

[c] A commercial equilibrium mixture of mono-, di-, and triglycerides prepared by reacting partially hydrogenated cottonseed oil with glycerine. It will be identified subsequently in this paper by the abbreviation CMDG.

[d] A partial ester of commercial stearic acid and prefabricated polyethylene glycol, which will be identified subsequently in this paper by the abbreviation PMS No. 1.

[e] A partial ester of commercial lauric acid, sorbitan, and mixed polyoxyethylene diols having an average chain length corresponding to 20 ethylene oxide units per mol of sorbitan. It will be identified subsequently in this paper by the abbreviation PSML.

substance used and had the following percentage composition: vitamin-free casein 18.0; sucrose 49.2; control or test substance 25.0; Jones and Foster *(11)* salt mixture 4.0; agar 2.0; vitamin A and D oil (2250 U.S.P. units of A and 300 U.S.P. units of D per g.) 1.0; vitamin mixture[f] 0.8. After 6 weeks, the diets were modified by increasing the casein to 24% at the expense of sucrose and by adding 0.3 g. of L-cystine to each 100 g. of finished diet.

In the second experiment weanling hamsters (6 males, 4 females in each group) were fed the 18% casein synthetic diets containing 25% of lard or PMS No. 1 for 22 weeks. In the third experiment, also with hamsters, these synthetic diets were modified to contain 24% of casein and either 15% lard or PMS No. 1, with corresponding changes in sucrose to adjust to 100%; in addition 0.3 g. of L-cystine was added to each 100 g. of the finished diet. Each group in this third experiment contained 3 male and 7 female weanling hamsters which were fed for 39 weeks.

In the fourth experiment hamsters were given diets containing 5, 10, or 15% levels of lard, "lard" mono- and diglycerides (LMDG)[g], PMS No. 1, polyoxyethylene monostearate (PMS No. 2)[h], or PSML which had been baked into a "bread" before incorporation into the experimental diet. One hundred and seventy weanling male hamsters (4 to 6 weeks of age) were conditioned for 15 days on a "bread" diet of the following percentage composition: white flour 65.7; hydrogenated vegetable shortening 15.0; lactalbumin 9.0; Jones and Foster *(11)* salt mixture 4.0; sucrose 2.0; fresh yeast 1.5; wheat germ oil 1.0; vitamin A and D oil (2250 U.S.P. units of A and 300 U.S.P. units of D per g.) 1.0; vitamin mixture[i] 0.8. After the conditioning period to insure good acceptance of the diets and to eliminate those hamsters not eating the bread diet readily, the hamsters were distributed into 15 uniform groups of 10 each.

In the experimental diets the 15% hydrogenated shortening was replaced by 5, 10, or 15% levels of the control or test substance. To complete the adjustment of the diet composition 10, 5, or 0% additional white flour, respectively, was added.

The bread components of the diets were prepared by adding water[j] and making doughs of all the ingredients except the vitamin A and D oil, wheat germ oil, vitamin mixture and ¾ of the salt mixture. These doughs were mixed, handled, and baked at 405°F. for 35 min. in conventional bakery equipment; the resulting "breads" were

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[f] Composition of vitamin mixture in each 100 g. of diet = choline chloride (Merck) 100 mg.; 2 methyl naphthoquinone 1.0 mg.; thiamine hydrochloride, riboflavin, and pyridoxine hydrochloride each 0.8 mg.; d-calcium pantothenate 4.4 mg.; niacin 4.0 mg.; pteroylglutamic acid 25 gamma; biotin 10 gamma; Wilson liver concentrate 1:20 N.F. (IX) 0.7 g. The vitamins were premixed with 19.2 g. of the sucrose for addition to the diets.

[g] A commercial equilibrium mixture of mono-, di-, and triglycerides prepared by reacting a mixture of 95% lard and 5% hydrogenated vegetable oil with glycerine. It will be identified subsequently in this paper by the abbreviation LMDG.

[h] This substance, which will be identified subsequently in this paper by the abbreviation PMS No. 2, is a partial ester of commercial stearic acid and mixed polyoxyethylene diols produced by the direct action of 8 mols of ethylene oxide with one mol of commercial stearic acid. It has been reported to contain approximately 44% free fatty acid and 53% oxyethylene. It is not a "monostearate," but rather an equilibrium mixture of mono- and di-esters and the corresponding free diols, containing approximately 19% free polyols, 33% mono-esters, and 48% di-esters. A minute quantity (0.04 to 0.06%) of ethylene glycol is present *(4)*.

[i] The same vitamin mixture described under footnote f modified by adding 10 mg. each of para amino benzoic acid and inositol to each 100 g. of the diet. The vitamin mixture was not premixed with sucrose.

[j] To make a dough, the following quantities of water were added for each 100 g. formula weight of the respective diets; for 5% diets, 56.6 g. water; for 10%, 53.3 g.; for 15%, 50.0 g.

sliced and air dried at room temperature. The dried bread components[4] were finely ground, and the remaining ingredients listed above were then thoroughly mixed into them to make the finished "bread" diets. These diets kept well and were eaten readily by the hamsters.

To insure against rancidity fresh batches of the diets were prepared frequently (at least monthly). Reserve supplies were stored at about −10°F. until needed, and the containers from which the diets were weighed were held at 45-50°F. while not actually in use.

All animals in all experiments were individually caged in the same animal room which was maintained at 80°F. ± 2° and at a relative humidity averaging about 45%. Food and water were offered ad libitum. All animals were inspected daily and were fed and weighed twice weekly. Individual food disappearance records were kept for all animals throughout the experimental periods. Since waste was usually small and was intermixed frequently with the diarrheal stools characteristic of most experimental groups, it was not recovered for weighing. Hamsters were purchased from the Haskins Rabbitry, St. Louis, Missouri.

RESULTS

Average gains, food consumption, and mortality for the first experiment (rats) are presented in Table 1. The control animals fed diets containing lard or CMDG appeared normal in all respects. Growth was good and almost identical throughout the experiment for these 2 groups. The animals fed diets containing PMS No. 1 or PSML gained significantly less than the control groups. The PMS No. 1 group fell behind during the first week; the growth rate of the PSML group paralleled that of the controls for the first 6 weeks, but decreased rather suddenly during the 7th week.

TABLE 1

Weight gain, food intake, and mortality of rats fed diets containing 25% levels of lard, cottonseed oil mono- and diglycerides (CMDG), polyoxyethylene monostearate (PMS No. 1), or polyoxyethylene sorbitan monolaurate (PSML). (Experiment 1)

Dietary variable	Lard	CMDG	PMS No. 1	PSML
No. of rats started	10	8	8	10
Av. initial wt.—g.	53.8	54.4	53.3	54.0
Av. gain—8 wks.—g.	237.5(9)[1]	227.0	153.2(6)	174.0(9)
21 wks.—g.	375.2(9)	386.1	285.8(5)	272.2(8)
Av. food eaten—8 wks.—g.	633(9)	604	650(6)	850(9)
21 wks.—g.	1759(9)	1753	2226(5)	2613(8)
Av. food/gain—8 wks.	2.67	2.66	4.24	4.89
21 wks.	4.69	4.54	7.78	9.61
No. died[2]—21 wks.	0[3]	0	2[3] (1st—1) (2nd—1)	1[3] (19th)

[1] Numbers in parentheses following gains designate the number of animals which were averaged to obtain the average gain. This procedure will be used consistently in all tables for all averages whenever the number per group differs from the number started.

[2] Figures in parentheses following the number died designate the weeks in which deaths occurred and the number dying during the week. This method is used consistently in all tables.

[3] One animal was sacrificed for examination and/or exploratory analyses in each of the following groups: lard, 3rd week; PMS No. 1, 13th week; PSML, 3rd week.

[4] Baking of the "bread" component of the diets resulted in certain losses which are inherent in bread baking, e.g., fermentation losses (carbon dioxide, alcohol), and mechanical losses which were associated with mixing and handling the dough and drying and grinding the "bread." Thus, each 100 g. formula weight of diet actually yielded an average of 93.4 g. of finished "bread" diet as fed. The actual concentration of control or test substances in the "bread" diet as fed therefore may have been increased slightly but uniformly for this reason, but not to exceed 5.3, 10.7, or 16.1% for the three dietary levels. For convenience, the formulation percentages have been used throughout this paper.

In addition to the effects observed on growth and food consumption, all rats in the PSML group showed severe diarrhea which began on or before the third day and eventually led to extensive inflammation and ulceration of the region around the anus. Stools from these rats were light brown in color and of a liquid consistency. Occasional checks with Hydrion pH paper indicated a pH of about 8 for the feces of the rats fed PSML, and about pH 6 for the feces of the rats in all other groups. All rats fed PMS No. 1 consistently showed light brown to grayish-white fecal pellets considerably larger in size than the small black pellets of the rats fed either lard or CMDG. The pooled feces of all rats in each group were examined for occult blood (using the benzidine reaction) on 3 to 5 occasions at various times during the course of the experiment. When a positive or doubtful reaction was encountered, the feces from each individual rat in the group were examined on one or two additional occasions. The feces of each rat fed PMS No. 1 consistently gave a strongly positive benzidine reaction for blood throughout each pellet. Those from rats in the other groups were consistently negative.

It was observed that all rats fed PMS No. 1 or PSML showed a tendency toward increased water consumption and urine excretion (more rapid emptying of water bottles and relatively large accumulations of urine on the water-proof waxed paper used routinely under all cages). This qualitative observation regarding water intake was confirmed by measuring carefully the water consumption for several days during the 11th week. The daily average water consumptions for rats fed lard, PMS No. 1 or PSML were, respectively, 62, 139, and 242 ml. per kilogram body weight.

All rats surviving at the end of 21 weeks were sacrificed for gross and histopathological examination. These findings are described in detail in a separate paper *(2)*.

At the time of sacrifice, the blood, visible body fat depots, and skinned carcasses (head, entire gut, and visible fat removed) were analyzed for polyoxyethylene glycols by the method of Shaffer and Critchfield *(26)*. Three rats each were analyzed from the groups fed the diets containing PMS No. 1 or PSML and one each (blood only) from the groups fed diets containing lard or CMDG. This exploration revealed polyoxyethylene glycols in the blood (6 to 23 mg. per cc.) of the rats ingesting PMS No. 1 or PSML diets; only very small, probably insignificant traces in the skinned carcasses; and none in the fat depots. The control rats fed lard or CMDG showed no polyoxyethylene glycols in the blood.

Synthetic diets containing 25% PMS No. 1 or 25% lard from the same batches prepared for the rats in experiment 1 described above were offered to weanling hamsters at the same time the rat feeding test was started. All of the 10 hamsters offered the 25% PMS No. 1 diet ate very poorly and all 10 were dead within two weeks. Of 10 hamsters started on the diet containing 25% lard, two ate very little of it and died within the first week, but the other 8, after an initial weight loss during the first few days on the diet, did very well.

It was not clear whether the early death of all the hamsters started on the 25% PMS No. 1 diet was due to voluntary starvation alone or to some other harmful effect of PMS No. 1. For this reason, the 8 surviving hamsters fed the lard diets were distributed into two subgroups of 4 each at the end of the 4th week. One subgroup was continued on the lard diet, and the hamsters continued to thrive. The other subgroup was changed to the PMS No. 1 diet and, although they ate more of the diet than those started initially on the PMS No. 1 diet, their food intake was still less than that of the hamsters continued on the lard diet. Three were dead within 4 weeks; all 4 were dead within 9 weeks after making the change from the lard to the PMS No. 1 diet. After 21 weeks the 4 hamsters that had been continued on the lard diet were sacrificed and examined for any gross pathology. They were all well nourished, with good distribution of body fat, and the organs and tissues appeared to be normal on gross inspection.

Similar results were obtained in the third experiment in which weanling hamsters were fed synthetic diets containing 15% of lard or of PMS No. 1. The 10 hamsters fed the lard diet grew well, but those fed the PMS No. 1 diet did poorly, and all 10 were dead within 15 days. As before, the lard-fed controls were divided into two subgroups of 5 each, one of which was continued on the lard diet, while the other was transferred to the PMS No. 1 diet at the end of 4 weeks. The hamsters remaining on the lard diet continued to grow to adult size; those transferred to the PMS No. 1 diet failed to maintain the same rate of gain and all died during a period of 33 weeks.

TABLE 2

Body weight, food intake, and mortality of hamsters fed diets containing 25% (Experiment 2) or 15% (Experiment 3) of lard or polyoxyethylene monostearate (PMS No. 1) for four weeks.

Dietary level	25%		15%	
Dietary variable	Lard	PMS No. 1	Lard	PMS No. 1
No. hamsters started	10	10	10	10
Av. initial wt.—g.	49.3	49.9	61.2	60.0
Av. wt.—1 wk. —g.	51.4(8)	38.3(6)	66.4	44.1(7)
—4 wks.—g.	85.4(8)	all dead	80.7	all dead
Av. food eaten—1 wk. —g.	24(8)	6(6)	37	9(7)
—4 wks.—g.	127(8)	all dead	144	all dead
Mortality—4 wks.	2(1st—2)	10(1st—4) (2nd—6)	0	10(1st—3) (2nd—6) (3rd—1)

The average body weights, food intakes, and the mortality of hamsters fed synthetic diets containing 25 or 15% levels of lard or PMS No. 1 are shown in Tables 2 and 3.

Post-mortem examination of the 33 hamsters which died yielded no unusual, consistent gross pathological findings. Five of the 10 hamsters which died so quickly in each group when fed the 25% or 15% PMS No. 1 diets did show empty and contracted gastrointestinal tracts. Three of the 5 hamsters which died when changed after 4 weeks from a 15% lard diet to a 15% PMS No. 1 diet showed bladder involvements such as hypertrophy of the bladder wall and the presence of bloody urine. The three surviving hamsters in the subgroup continued on the 15% lard diet were sacrificed for gross and histopathological examination after 37-39 weeks. Two of these animals showed no pathological changes while the third showed obstructive nephropathy, enlargement of the spleen, and inflammation of the gastrointestinal tract.

The average weight, food consumption, and mortality of the hamsters fed bread diets containing 5, 10, or 15%, respectively, of lard, LMDG, PMS No. 1, PMS No. 2, or PSML are shown in Tables 4, 5, and 6. The growth of the hamsters fed 5% of PMS No. 1, PMS No. 2, or PSML was significantly retarded, tending to fall behind that of the corresponding lard or LMDG-fed group after the 8th week. The 10 and 15% levels of lard or LMDG in the diet led to even better growth and adult size, but

TABLE 3

Body weight, food intake, and mortality of hamsters fed diets containing 25% or 15% of lard or polyoxyethylene monostearate (PMS No. 1), after preliminary feeding of lard (Experiments 2 and 3 after subdivision).

Dietary level	25%		15%	
Dietary variable	Lard	PMS No. 1	Lard	PMS No. 1
No. hamsters started	4	4	5	5
Av. initial wt.—g.	84.0	86.7	79.4	82.0
Av. wt.— 1 wk. —g.	89.5	74.5	82.0	79.2
— 4 wks.—g.	102.3	82(1)	91.2	81.4
— 8 wks.—g.	111.0	62(1)	94.6	89.3(4)
—17 wks.—g.	119.5	all dead	99.4	93.0(3)
—32 wks.—g.	.....	.....	96.8(4)	86(1)
Av. food eaten—1 wk. —g.	31	16	35	23
—4 wks.—g.	135	97(1)	137	116
—8 wks.—g.	270	214(1)	272	255(4)
Mortality to end of experiment[1]	0	4(1st—1) (2nd—1) (4th—1) (9th—1)	2(31st—1) (34th—1)	5(8th—1) (13th—1) (28th—2) (33rd—1)

[1] Thirty-five weeks and five days.

TABLE 4

Body weight, food intake, and mortality of hamsters fed "bread" diets containing 5% of lard, lard mono- and diglycerides (LMDG), polyoxyethylene monostearates (PMS No. 1 and PMS No. 2) and polyoxyethylene sorbitan monolaurate (PSML). (Experiment 4).

Dietary variable	Lard	LMDG	PMS No. 1	PMS No. 2	PSML
Av. initial wt.[1]—g.	68.3	72.4	70.7	69.8	71.4
Av. wt.—					
1 wk. —g.	69.2	77.6	73.7	70.4	72.4
4 wks.—g.	80.5	87.0	79.4	79.4(9)	79.3
8 wks.—g.	88.3	92.0	78.8	87.1(9)	84.7
16 wks.—g.	97.1	97.9	86.0(9)	91.3(9)	90.1
27 wks.—g.	98.3	98.0	95.6(8)	92.4(8)	91.1(9)
Av. food intake—					
1 wk. —g.	32	38	34	38	31
8 wks.—g.	279	287	281	313(9)	309
27 wks.—g.	957	973	1009(8)	1001(8)	1053(9)
Mortality—39 wks.	0	0	3(11th—1) (17th—1) (37th—1)	2(2nd—1) (22nd—1)	1(21st)

[1] Ten hamsters were started in each group.

the 15% level of PMS No. 1 or PSML decreased the growth and final size, despite the increased food intake.

Sacrifice of the surviving hamsters fed the bread diets was begun during the 28th week and completed during the 39th week. Average weights and food intakes were not tabulated beyond 27 weeks because of the reduction (for histopathological examination) in the number of hamsters surviving in each group after this time. Complete gross pathological examinations were made on the 39 hamsters that died and on the 111 surviving hamsters that were sacrificed for detailed histopathological examination; these observations are reported elsewhere *(2)*.

Diarrhea was observed on or before the third day in all of the hamsters fed PMS No. 1, PMS No. 2, or PSML at all 3 levels of feeding but in greatest degree at the higher levels. PSML consistently produced the most severe diarrhea. The hamsters fed

TABLE 5

Body weight, food intake, and mortality of hamsters fed "bread" diets containing 10% of lard, lard mono- and diglycerides (LMDG), polyoxyethylene monostearates (PMS No. 1 and PMS No. 2) and polyoxyethylene sorbitan monolaurate (PSML). (Experiment 4).

Dietary variable	Lard	LMDG	PMS No. 1	PMS No. 2	PSML
Av. initial wt.[1]—g.	71.7	71.8	72.7	72.4	73.1
Avt. wt.—					
1 wk. —g.	76.3	75.2	73.2	70.2	70.6
4 wks.—g.	88.0	87.5	79.6	76.7	78.7(6)
8 wks.—g.	96.9	95.0	84.6	81.0(8)	85.5(6)
16 wks.—g.	103.1	101.8	89.7	85.9(8)	97.8(4)
27 wks.—g.	107.7	105.0	93.5	90.1(7)	100.5(2)
Av. food intake—					
1 wk. —g.	36	35	32	32	29
8 wks.—g.	280	282	301	291(8)	300(6)
27 wks.—g.	1000	987	1051	1032(7)	1105(2)
Mortality—39 wks.	1(29th)	0	0	3(4th—1) (8th—1) (21st—1)	8[2]

[1] Ten hamsters were started in each group.
[2] Deaths occurred at following times: 2nd wk.—2; 3rd—2; 9th—1; 13th—1; 26th—1; 27th—1.

TABLE 6

Body weight, food intake, and mortality of hamsters fed "bread" diets containing 15% of lard, lard mono- and diglycerides (LMDG), polyoxyethylene monostearates (PMS No. 1 and PMS No. 2) and polyoxyethylene sorbitan monolaurate (PSML). (Experiment 4).

Dietary variable	Lard	LMDG	PMS No. 1	PMS No. 2	PSML
Av. initial wt.[1]—g.	72.4	71.7	71.9	72.6	72.2
Av. wt.—					
1 wk. —g.	75.4	76.1	67.1	66.7	62.0
4 wks.—g.	83.5	86.0	71.3	71.1	57.0(4)
8 wks.—g.	92.4	93.9	75.0(8)	76.0	54.0(2)
16 wks.—g.	102.7	99.6	79.5(6)	84.2(6)	55(1)
27 wks.—g.	107.4	101.6	83.0(5)	93.3(4)	56(1)
Av. food intake—					
1 wk. —g.	34	34	29	29	21
8 wks.—g.	268	263	313(8)	306	261(2)
27 wks.—g.	928	920	1078(5)	1072(4)	1066(1)
Mortality—39 wks.	1(31st)	0	5[2]	6[3]	9[4]

[1] Ten hamsters were started in each group.
[2] Deaths occurred at following times: 6th wk.—1; 7th—1; 10th—1; 15th—1; 21st—1.
[3] Deaths occurred at following times: 11th wk.—1; 13th—2; 16th—1; 21st—1; 25th—1.
[4] Deaths occurred at following times: 2nd wk.—4; 3rd—2; 6th—1; 8th—1; 12th—1.

the 10 and 15% levels of PSML showed tense, bloated abdomens, and there was evidence also of inflammation and ulceration of the anal region in the hamsters fed at the 15% level.

The hamsters fed PMS No. 1, PMS No. 2, and PSML showed poor hair coat and general unthrifty appearance even at the 5% level of feeding; this was more pronounced at the 10% level and quite marked at the 15% level.

Hemorrhages from the genitourinary tract and hematuria began to appear in many of the hamsters during the 7th week. Thereafter inspections were made daily to determine the frequency and severity of the bleeding. The hamsters fed PMS No. 1 and PMS No. 2 bled most frequently, those fed PSML bled to a lesser extent, and those fed lard of LMDG bled only rarely. Confirmation with the benzidine reaction was used only in those few cases where it was necessary to distinguish between blood and fecal pigments on the paper under the mesh-bottom cages. For individual hamsters the bleeding occurred as intermittent episodes of varying severity, usually lasting from one to several days, followed by an interval of one or more days (or even weeks in many instances) in which there was no gross indication of bleeding.

For the entire experimental period there were, in all, 22 instances of bleeding (7 slight, 8 moderate, 7 severe) involving 9 lard-fed hamsters; 36 instances (9 slight, 11 moderate, 16 severe) involving 7 hamsters fed LMDG; 300 instances (118 slight, 74 moderate, 108 severe) involving 25 hamsters fed PMS No. 1; 274 instances (74 slight, 75 moderate, 125 severe) involving 22 animals fed PMS No. 2; and 46 instances (23 slight, 16 moderate, 7 severe) involving 8 of the 12 surviving hamsters fed PSML.

DISCUSSION

Results of the first experiment demonstrate clearly that, under the experimental conditions described, the ingestion of 25% of either PMS No. 1 or PSML in a synthetic diet markedly reduces both the rate of growth and final adult size of the rats when compared with the control groups fed identical levels of either lard or CMDG. This occurred in spite of food intakes for the PSM No. 1 or PSML groups which greatly exceeded those of the lard or CMDG controls.

It seems probable that the markedly increased food intakes observed for both the PMS No. 1 and PSML groups demonstrate a lowered caloric density for these diets. Although at the time this work was undertaken,

there was no information available regarding the caloric contribution of the polyoxyethylene portion of esters such as PMS No. 1 or PSML, it has now been reported that such polymers are not utilized for calories *(4)*. On the basis of a full utilization of the fatty acid portion of these substances and no utilization of the remainder for calories, the calculated caloric densities of the PSML and PMS No. 1 diets are 3.1 and 3.8 Cals. per g., respectively, as compared with 5.0 for the lard and 4.9 for CMDG diets. Using these values, the calculated calories consumed per gram of gain by the PMS No. 1 and PSML groups are actually greater than those calculated for the control groups. Thus, caloric insufficiency does not seem to be a likely explanation for the poor gains observed for the PMS No. 1 and PSML-fed groups. Others *(8, 9, 24)* have evaluated the effect of combining nutritionally inert material (Celluflour) with triglyceride to simulate calorically the polyoxyethylene monostearate preparations. Poorer growth was obtained with the diets containing the polyoxyethylene preparations than with those of similar caloric densities containing the Celluflour, thus indicating that the polyoxyethylene components were not biologically inert.

It was not clear whether the death of all hamsters within 2 weeks when fed 25% or 15% levels of PMS No. 1 in synthetic diets (Experiments 2 and 3) was due to starvation because of dislike for the diet or to a harmful effect of PMS No. 1. Even after a 4-week preliminary feeding of lard diets, the hamsters subsequently transferred to PMS No. 1 diets still failed to eat enough to compensate for the lower caloric density of the latter diets. Schweigert, McBride, and Carlson *(24)* have reported marked growth inhibition in hamsters fed 15% levels of two polyoxyethylene monostearates (one of which was PMS No. 1), even though the food consumption of their animals exceeded that of the lard-fed controls.

An attempt was made in Experiment 4 to design a diet which would meet all the known nutritional requirements of the hamsters and which would parallel the actual conditions of use of the experimental materials to the extent of preparing the diets as "bread." This would include any possible effects resulting from heat in the presence of other ingredients in the "bread."

The lower caloric intakes observed for the experimental hamsters in the earlier experiments with the synthetic diets were eliminated in this last experiment with hamsters fed the "bread" diets. The calculated caloric intakes[1] of the experimental groups were quite similar to those of the control groups when allowance is made for the utilization of the calories supplied by the fatty acid part of the polyoxyethylene esters, but the calories consumed per gram gained were markedly higher for the experimental groups than for the control animals. The latter would be true even if the fatty acid portions were not utilized for calories.[m]

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[1] Calculated caloric densities (Cals. per gram) for the various diets are, in the order following, lard, LMDG, PMS No. 1, PMS No. 2, and PSML; 5% level = 3.7, 3.7, 3.6, 3.6, 3.4; 10% level = 4.0, 4.0, 3.6, 3.6, 3.3; 15% level = 4.3, 4.2, 3.6, 3.6, 3.2.

[m] Calculated caloric densities of the experimental diets are as follows if assumption of no utilization of the polyoxyethylene esters is made: 5% levels = 3.3; 10% levels = 3.2; 15% levels = 3.0 Cals. per gram.

No completely adequate explanation is suggested by the data for the pronounced tendency toward bleeding from the genitourinary tract seen in the hamsters fed bread diets containing PMS No. 1, PMS No. 2, or PSML, and to a relatively slight extent in the control animals fed lard or LMDG. Bleeding was seldom seen in the hamsters fed synthetic diets containing PMS No. 1, perhaps arguing against a general inherent tendency toward genitourinary bleeding in the hamsters. The good growth, low mortality, thrifty appearance, and general lack of significant gross or histopathological changes *(2)* in the control animals after 28 to 39 weeks argues against dietary insufficiency. Whatever the cause, there was marked aggravation of the condition by PMS No. 1, PMS No. 2, and to a lesser extent by PSML. The incidence of bleeding in the PSML-fed hamsters should be interpreted in the light of the fact that 11 of the 18 fatalities out of the original 30 hamsters fed PSML occurred early in the experiments before any bleeding had been observed in any of the animals.

The exact origin of the blood in the genitourinary tract was not located, although blood clots of varying sizes, some relatively large, were found in the bladders of several hamsters at autopsy. The pH of the urine did not seem unusually low, ranging from pH 5 to pH 6 in the few determinations made. A chronic inflammation somewhere in the genitourinary tract is considered a possibility; the passage or movement of small urinary calculi[a] is another.

The mortality data for the several groups of animals presented in the tables of data leave no doubt of the adverse effect of the PMS No. 1, PMS No. 2, and PSML diets upon survival. Specifically, there were 6 deaths during the course of the experiments out of a total of 98 animals (rats plus hamsters) which were started on the control diets containing lard or commercial equilibrium mixtures of mono-, di-, and triglycerides. On the other hand, there were 69 deaths out of a total of 137 rats plus hamsters which were started on or changed to the PMS No. 1, PMS No. 2, and PSML diets. Moreover, only 2 of the 6 deaths in the control groups occurred by the end of the 26th week, whereas 65 of the 69 deaths in the experimental groups had occurred by that time. Broken down further according to each control or experimental substance fed, the over-all mortality ratios were as follows: for lard 6/60; for mono- and diglycerides 0/38; for PMS No. 1 39/67; for PMS No. 2 11/30; and for PSML 19/40.

The general relationship between the ingestion of various levels of the polyoxyethylene preparations and decreased growth, decreased thriftiness, increased mortality, bleeding from the genitourinary tract, increased gross and histopathology *(2)* leaves little doubt of their harmfulness to rats and hamsters under the conditions of these experiments.

SUMMARY

The effects of ingesting various levels of polyoxyethylene-derived surface active preparations (two polyoxyethylene stearates and polyoxyethylene sorbitan monolaurate) were observed in rats and hamsters with lard

[a] Six unusually large bladder stones were found and have been described in detail elsewhere *(2)*.

or mono- and diglycerides as control substances. In general, the animals fed the experimental substances showed:

1. Retardation of growth.
2. Decreased adult size.
3. Markedly decreased efficiency of food utilization.
4. Markedly increased water consumption in rats.
5. A generally unthrifty appearance.
6. Increased mortality.
7. Consistent moderate to severe diarrhea.
8. Hemorrhage from the genitourinary tract in hamsters.

The harmful effects observed in animals fed polyoxyethylene-derived surface active agents at dietary levels as low as 5% indicate that considerable caution should be exercised before such substances are used in foods for human consumption.

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THE ORAL TOXICITY AND PATHOLOGY OF POLYOXYETHYLENE DERIVATIVES IN RATS AND HAMSTERS[a]

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Despite the use of polyoxyethylene derivatives in a wide variety of food products, little has been published on the toxicological and microscopic findings after long-term feeding of these materials to establish their safety for use in food for human consumption. Wang, McBride, and Schweigert *(9)* reported on the adverse histological manifestations in hamsters after feeding polyoxyethylene monostearates for 10 weeks, and Harris *et al.* pointed out the harmful nutritional and pathologic effects of sorbitan monolaurate, polyoxyethylene sorbitan monolaurate, polyoxyethylene monolaurate, and polyoxyethylene monostearate when fed to rats for 59 and 70 days *(3)*, and when fed to hamsters for 68 days *(4)*.

Recently Krehl, Cowgill, and Whedon *(5)* published data on nondeleterious effects after feeding polyoxyethylene mono- and distearates, mono- and dioleates, and mono- and dilaurates for varying periods of time, including a maximum of 513 days for 1 group of rats and 367 days for several cats. In a study of the chronic toxicity of various bread additives to rats Graham, Teed, and Grice *(2)* fed polyoxyethylene-8-monostearate in a bread diet for one year, and Graham and Grice *(1)* fed the same compound in a synthetic ration for 32 weeks with no detectable effects.

The present paper deals with acute oral toxicity studies on a series of polyoxyethylene, sorbitan and polyoxyethylene sorbitan esters of fatty acids in hamsters, rats, and rabbits; with the effect of repeated daily doses of polyoxyethylene monostearates in hamsters; and with the gross and microscopic pathology after feeding polyoxyethylene monostearates and polyoxyethylene-20-sorbitan monolaurate at 25% levels in synthetic diets to rats for 21 weeks and at 5, 10, and 15% levels in bread diets to hamsters for 28-39 weeks.[b] One or more of the following were used as the control materials in the various experiments: lard, cottonseed oil, distilled water, lard mono- and diglycerides, cottonseed oil mono- and diglycerides, and cottonseed oil monoglycerides. The nutritional effects in these latter rat and hamster feeding studies have been reported *(6)*.

EXPERIMENTAL

Acute oral toxicity. The median lethal dose studies were performed on male rats (150-220 g.), on young adult hamsters, and on rabbits (3-4 kg.), all of which had

[a] Presented as Exhibit 366, Ice Cream Hearings, Docket FDC 34A, 1952.

[b] These materials were purchased on the open market for use in food products and contained traces of unidentified impurities. They will be designated by chemical rather than trade names, with an average figure indicated for the mols of alkylene oxide per mol of sorbitan or of fatty acid. The abbreviations that will be used hereafter are given in Table 1.

fasted 18 hours, with water *ad libitum*. Each animal was kept in a separate cage in an air-conditioned animal room maintained at 80° ± 2°F. and ca. 45% relative humidity. The materials tested were administered by stomach tube as liquids (in some cases warming on a water bath was required). Calculation of LD_{50} values was made by the method of Reed and Muench *(7)*.

Single doses (20 ml.) of the polyoxyethylene monostearates or distilled water were administered to a total of 27 fasting rabbits. After 24 hours these animals were sacrificed under ether anesthesia, and careful examination of the organs was made. The intact bladder was clamped off at the base and removed, and the bladder contents were drained into glass jars for inspection and analysis. The polyoxyethylene glycol content of the urine samples was determined by the method of Shaffer and Critchfield *(8)*.

Examination of animals and tissues. All of the rats and hamsters were assigned new autopsy numbers which were scrambled and bore no logical relation to the previous dietary history, so that each of the animals autopsied was an unknown to the individuals making the gross observations and to the pathologists who studied the slides. The pathologists were also unaware of the nature of the materials used in this study until their final written reports on microscopic findings had been made. In the case of the rats, portions of the kidney, bladder, liver, adrenal, stomach, jejunum, cecum, spleen, pancreas, heart and aorta, lung, testis, femoral marrow, mediastinum and mesenteric lymph node were fixed in 10% formalin, and blocked in paraffin. Sections 8 micra in thickness were stained with hematoxylin and eosin. The femoral marrow was teased out with a probe after fixation and processed in the same manner as the other tissues. Frozen sections of liver and kidney were stained with Sudan III. In the case of the hamsters, trimmed pieces of the following tissues were fixed in 10% formalin-Zenker: kidney, bladder, liver, adrenal, stomach, small intestine, cecum, spleen, pancreas, heart and aorta, lung, testis, and bone marrow. After processing, each tissue was imbedded in a separate block of 57° paraffin or 60-62° Tissuemat. Sections 6 or 8 micra thick were stained with hematoxylin and eosin and mounted in synthetic resin. For the study of fat, additional portions of kidney and liver from each hamster were fixed in 10% formalin, processed and frozen, cut at 12-16 micra, stained with Sudan Black or Scarlet Red and mounted in glycerine jelly or Apathy's fluid.

RESULTS

Acute oral toxicity. The LD_{50} data obtained after administration of single doses of a series of materials to various animal species and observation for one week are given in Table 1. Definite LD_{50} values were obtained for PMS No. 1, PMS No. 2, and PSML in the hamster and for PMS No. 1, PMS No. 2, the polyoxyethylene portion of PMS No. 2, PSML, polyoxyethylene-20-sorbitan monooleate, sorbitan monostearate, and glyceryl monolaurate in the rat. The following materials could be administered by stomach tube to the rat to the point of maximum distention of the stomach and overflowing into the duodenum (90 ml./kg.) without fatalities: cottonseed oil monoglyceride, cottonseed oil mono- and diglycerides, cottonseed oil, lard mono- and diglycerides, lard, and distilled water.

Sub-acute oral toxicity. Table 2 lists the results from repeated daily intubations of PMS No. 1, PMS No. 2, and distilled water for a period of 8 days. The body weights of the surviving hamsters were recorded for as long as 10 weeks after the 8th dose, and, despite an initial depression lasting a maximum of 7 days after administration of the last dose, they showed good recovery and subsequently were not significantly different from the body weights of the water-administered controls. An LD_{50} value calculated on the basis of 8 consecutive daily intubations in these hamsters was 1.36 ml./hamster/day for PMS No. 1 and 1.44 ml./hamster/day for PMS No. 2.

Effect of single doses on the rabbit. Single doses of PMS No. 1 or PMS No. 2 caused no obvious gross abnormalities in rabbits sacrificed 24 hours after administration of 20 ml. by stomach tube other than the urinary findings summarized in Table 3. In contrast to the normally cloudy, alkaline urine containing varying amounts of white, crystalline precipitate, as was the case for the controls which received distilled water, the urine from the PMS No. 1- or PMS No. 2-administered rabbits was clear, acidic, and contained no precipitate. The normally occurring precipitate was insoluble in water, dilute NaOH, and the common organic solvents but was soluble in dilute HCl with

TABLE 1

Acute oral toxicity studies in various animal species.

Material	Species	Number animals used	LD_{50}
			ml./kg.
Polyoxyethylene monostearate (PMS No. 1)	Hamster	38	19.5
Polyoxyethylene monostearate (PMS No. 1)	Rat	31	53.0
Polyoxyethylene monostearate (PMS No. 1)	Rabbit	35	>15.0
Polyoxyethylene-8-monostearate (PMS No. 2)	Hamster	59	27.0
Polyoxyethylene-8-monostearate (PMS No. 2)	Rat	79	64.0
Polyoxyethylene-8-monostearate (PMS No. 2)	Rabbit	21	>12.0
Polyoxyethylene portion of PMS No. 2	Rat	20	25.0
Polyoxyethylene-20-sorbitan monolaurate (PSML)	Hamster	47	18.0
Polyoxyethylene-20-sorbitan monolaurate (PSML)	Rat	57	36.7
Polyoxyethylene-40-sorbitan monostearate	Rat	16	>60.0
Polyoxyethylene-20-sorbitan monooleate	Rat	54	54.5
Sorbitan monostearate	Rat	35	31.0[1]
Glyceryl monolaurate	Rat	31	53.4
Cottonseed oil monoglyceride[2]	Rat	32	>90.0
Cottonseed oil mono- and diglycerides[3]	Rat	59	>90.0
Lard mono- and diglycerides[4]	Rat	56	>90.0
Cottonseed oil	Rat	24	>90.0
Prime steam lard	Rat	28	>90.0
Distilled water	Rat	19	>90.0

[1] Gm./kg.

[2] A monoester made by molecular distillation of cottonseed oil.

[3] A commercial equilibrium mixture of mono-, di-, and triglycerides prepared by reacting partially hydrogenated cottonseed oil with glycerine.

[4] A commercial equilibrium mixture of mono-, di-, and triglycerides prepared by reacting a mixture of 95% lard and 5% hydrogenated vegetable oil with glycerine.

considerable effervescence, and gave positive tests for carbonate, phosphate, and calcium. The 9 controls given intubations of distilled water showed no polyoxyethylene glycol in the urine; the 10 rabbits which received PMS No. 1 gave an average value of 5.85 mg. of polyoxyethylene glycol/ml.; and the 8 rabbits given PMS No. 2 showed an average value of 6.5 mg. polyoxyethylene glycol/ml. of urine.

Gross pathology. Because of the large number of experimental animals involved in these feeding experiments, described in detail elsewhere *(6)*, it is not feasible to list the many interesting pathological items noted for each.[c] The incidence of outstanding gross observations on rats fed at the 25% level is given in Table 4. The control rats fed cottonseed oil mono- and diglycerides or lard were very well nourished, slightly on the obese side, and manifested no unusual, consistent, gross abnormalities. There were no fatalities in the groups fed lard or cottonseed oil mono- and diglycerides; one of the lard-fed rats was sacrificed on the 19th day for use as a control for analyses of body fat. The PMS No. 1–fed rats likewise presented no outstanding gross pathology, although 2 of the original 8 rats in the group died during the experiment; another was sacrificed during the 13th week for gross pathological observation. The most striking changes occurred in the 8 rats fed PSML. The original number of rats in this group was 10; there was one fatality and one rat was sacrificed on the 19th day for the analyses of body fat. The PSML-fed rats showed unusually high incidence of the following gross findings: renal hypertrophy (100%), renal calculi (100%), ulceration of the tail at the region of the anus with acute inflammation and abundant granulation (100%), diarrhea (100%), urinary bladder calculi (75%), marked enlargement of the

[c] The detailed gross and microscopic findings noted in 30 rats and 111 hamsters, including descriptions of more than 2100 fixed tissues, were submitted as Exhibit 398, Ice Cream Hearings, Docket FDC 34A, 1952.

TABLE 2

Effect of eight repeated daily doses of polyoxyethylene monostearate in the hamster.

Material	Number hamsters	Daily oral dose	Result	
			Mortality ratio[1]	Notes
		ml.		
Polyoxyethylene monostearate (PMS No. 1)....	10	0.5	0/10	All survived
Polyoxyethylene monostearate (PMS No. 1)....	18	1.0	4/18	1 dead after 2 doses; 2 dead after 3 doses; 1 dead after 4 doses
Polyoxyethylene monostearate (PMS No. 1)....	8	2.0	8/8	6 dead after 1 dose; 1 dead after 2 doses; 1 dead after 3 doses
Polyoxyethylene monostearate (PMS No. 1)....	8	4.0	8/8	All dead after 1 dose
Polyoxyethylene-8-monostearate (PMS No. 2)	10	0.5	1/10	1 dead after 3 doses
Polyoxyethylene-8-monostearate (PMS No. 2)	10	1.0	0/10	All survived
Polyoxyethylene-8-monostearate (PMS No. 2)	10	2.0	10/10	4 dead after 1 dose; 1 dead after 2 doses; 2 dead after 3 doses; 1 dead after 5 doses; 2 dead after 8 doses
Distilled water.....	5	1.0	0/5	All survived
Distilled water.....	11	2.0	0/11	All survived

[1] Number animals dead/number animals dosed.

cecum (75%), etc. Indeed PSML caused a high incidence of all ten of the gross pathological findings listed in Table 4.

The fatalities during the course of the experiment on hamsters were distributed as follows: lard (2/30), lard mono- and diglycerides (0/30), PMS No. 1 (8/30), PMS No. 2 (11/30), and PSML (18/30). Both of the lard and one of the PMS No. 1 deaths occurred between the 28th and 39th weeks, the period during which all 111 surviving hamsters were being sacrificed according to the schedule mentioned previously.

Of the two lard-fed hamsters that died, one showed a hemorrhagic area in the wall of the urinary bladder. The eight hamsters being fed PMS No. 1 that died during the experiment had thickened, hemorrhagic bladders (5/8), hemorrhagic kidneys (4/8), and distended ceca filled with material giving a positive benzidine test for blood (4/8). Such frequent incidence of definite gross abnormalities as the following occurred in the hamsters fed PMS No. 2 that died: thickened, hemorrhagic urinary bladders (6/11),

TABLE 3

Effect of single doses of polyoxyethylene monostearates on the urine of the rabbit.

Material	No. of rabbits	Single oral dose	Urine				
			Av. vol.	Av. Sp. gr.	Av. pH	Av. polyoxyethylene glycol	Appearance
		ml.	ml.			mg./ml.	
Polyoxyethylene monostearate (PMS No. 1)	10	20	138	1024	5.45	5.85	9/9 clear urine; 1 bladder empty
Polyoxyethylene-8-monostearate (PMS No. 2)	8	20	56	1022	4.98	6.50	6/6 clear urine; 2 bladders empty
Distilled water	9	20	70	1040	7.24	0	9/9 cloudy urine

discolored hemorrhagic kidneys (7/11), hydronephrosis (3/11), and positive benzidine test for blood on the contents of the large bowel (5/11). The hamsters that were being fed PSML died earlier and in greater number than those in other groups. Indeed 14 of the total of 18 deaths in these hamsters occurred during the first twelve weeks on test. Despite the early fatalities in the PSML-fed groups, 10/18 of these dead animals still had developed hemorrhagic, distended ceca containing material which gave a positive benzidine test for blood.

The incidence of gross pathological findings in the 111 hamsters sacrificed for microscopic study is given in Table 5. There was no marked or unusual gross pathology in the control hamsters fed lard or lard mono- and diglycerides. For PMS No. 1 at all three levels combined 8/22 showed small testes, 13/22 had enlarged ceca, and 10/22 manifested a peculiar, brown discoloration of the liver. Particularly outstanding for the PMS No. 2–fed hamsters was the high incidence of markedly enlarged ceca (8/19). Of the surviving hamsters fed PSML, 8/12 had small testes, 8/12 had large, distended ceca, and 6/12 showed very pale (almost white), distorted, granular kidneys whose surfaces resembled that of a raspberry, suggestive of nephrosclerosis.

Only 1/28 of the control hamsters fed lard showed any diarrhea at the time of sacrifice; during the entire 28-39 weeks of feeding 2/30 had shown loose stools. Of the 30 control hamsters fed lard mono- and diglycerides, none showed any evidence of diarrhea from the beginning of the experiment to the time they were sacrificed. The incidence of liquid excreta at the time of sacrifice of the surviving hamsters fed PMS

TABLE 4

Summary of incidence of outstanding gross pathological findings on rats fed lard, cottonseed oil mono- and diglycerides, polyoxyethylene monostearate (PMS No. 1), and polyoxyethylene-20-sorbitan monolaurate (PSML) at the 25% level for 21 weeks (values in per cent of animals affected).

Gross pathological finding	Lard (9)[1]	Cottonseed oil mono- and diglycerides (8)[1]	PMS No. 1 (5)[1]	PSML (8)[1]
	%	%	%	%
Small testes	0	0	20	38
Stones in urinary bladder	0	12	0	75
Thickened bladder wall	0	0	0	38
Enlarged kidney	11	0	0	100
Stones in kidney	0	0	0	100
Enlarged spleen	22	25	0	50
Enlarged cecum	0	0	0	75
Ulceration in stomach	0	0	20	25
Ulceration of tail	0	0	0	100
Diarrhea	0	0	0	100

[1] The figures in parentheses denote the numbers of rats sacrificed for study of gross and microscopic pathology.

TABLE 5

Summary of incidence of outstanding gross pathological findings on hamsters fed 5, 10, and 15 percent levels of lard, lard mono- and diglycerides, polyoxyethylene monostearates (PMS No. 1 and PMS No. 2) and polyoxyethylene-20-sorbitan monolaurate for 28-39 weeks (in per cent of animals affected).

Dietary variable	No. hamsters sacrificed[1]	Diarrhea at time of sacrifice	Small testes	Thickened bladder walls	Distorted abnormal kidneys	Enlarged ceca	Brown discoloration of livers
		%	%	%	%	%	%
5% lard	(10)	10	10	10	0	10	0
10% lard	(9)	0	11	11	0	0	22
15% lard	(9)	0	22	0	0	0	0
Incidence at all 3 levels		4	14	7	0	4	7
5% lard mono- and diglycerides	(10)	0	0	20	0	20	0
10% lard mono- and diglycerides	(10)	0	30	0	0	0	20
15% lard mono- and diglycerides	(10)	0	30	10	0	0	20
Incidence at all 3 levels		0	20	10	0	7	13
5% polyoxyethylene monostearate (PMS No. 1)	(7)	57	14	28	14	0	14
10% polyoxyethylene monostearate (PMS No. 1)	(10)	70	50	30	30	90	60
15% polyoxyethylene monostearate (PMS No. 1)	(5)	60	40	20	60	80	60
Incidence at all 3 levels		64	36	27	32	59	45
5% polyoxyethylene-8-monostearate (PMS No. 2)	(8)	25	25	25	13	13	25
10% polyoxyethylene-8-monostearate (PMS No. 2)	(7)	71	43	14	71	43	14
15% polyoxyethylene-8-monostearate (PMS No. 2)	(4)	100	25	50	50	25	25
Incidence at all 3 levels		58	32	26	42	26	21
5% polyoxyethylene-20-sorbitan-monolaurate (PSML)	(9)	67	56	22	44	56	33
10% polyoxyethylene-20-sorbitan-monolaurate (PSML)	(2)	100	100	0	50	100	0
15% polyoxyethylene-20-sorbitan-monolaurate (PSML)	(1)	100	100	0	100	100	100
Incidence at all 3 levels		75	67	17	50	67	33

[1] The original number of hamsters in each group was 10, and the difference represents those that died during the experiment.

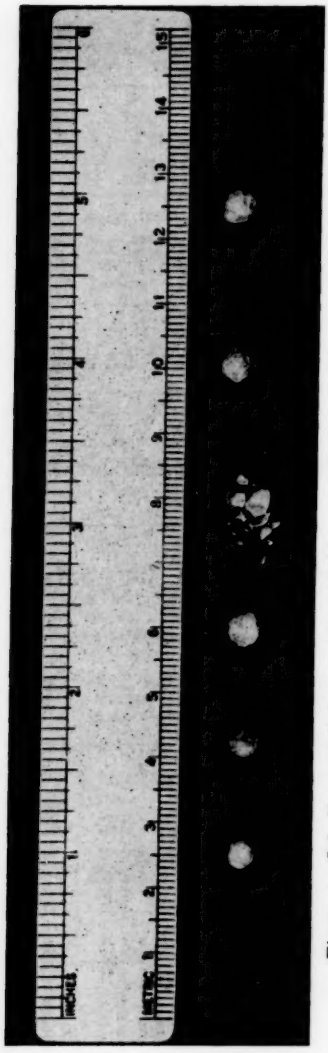

Figure 1. Calculi removed from urinary bladders of hamsters fed polyoxyethylene monostearates.

Stone number	Material fed	Days on diet	Diameter	Dry wt.	Remarks
			mm.	mg.	
1	5% PMS No. 1 in bread diet	74	5.0	70	Animal died
2	10% PMS No. 1 in synthetic diet	199	5.5	94	Animal died
3	15% PMS No. 2 in bread diet	169	5.5	53	Animal died
4	15% PMS No. 2 in bread diet	147	5.0	62	Animal died
5	10% PMS No. 1 in bread diet	259	5.0	69	Sacrificed *in extremis*
6	10% PMS No. 1 in bread diet	260	4.5	69	Sacrificed after X-ray demonstration of stone

No. 1 was 14/22, although 30/30 had a previous long history of moderate to severe diarrhea throughout the experimental period. Of the surviving PMS No. 2–fed hamsters 11/19 manifested varying degrees of diarrhea when sacrificed, though all 30 of the original animals had shown continued moderate to very severe diarrhea for the duration of the experiment. Of the 12 survivors from the original group of 30 hamsters which had been ingesting PSML, 9/12 showed diarrhea when sacrificed, although 30/30 of these animals had consistently produced the most severe diarrhea during the course of this experiment.

Extremely large calculi were found in the urinary bladders of five of the hamsters which had been fed PMS No. 1 and PMS No. 2 in bread diets. A sixth bladder concretion was found in one of a few hamsters which had been maintained for several months in an exploratory experiment on a synthetic diet containing 10% PMS No. 1. All six of these stones are shown in Figure 1. The fourth stone was crushed sometime after removal and proved to be primarily calcium phosphate.

Histopathology. The incidence of outstanding microscopic observations in the rats, fed at the 25% level, is summarized in Table 6. For lard there was a high incidence

TABLE 6

Summary of per cent incidence of outstanding histopathological findings on rats fed at the 25% level for 21 weeks (values are per cent of animals affected).

Histopathological finding	Dietary variable			
	Lard (9)[1]	Cottonseed oil mono- and diglycerides (8)[1]	PMS No. 1 (5)[1]	PSML (8)[1]
	%	%	%	%
Atrophy of testicular tubules	0	33	50	100
Decreased spermatogenesis	0	0	0	86
Calcified concretions in kidney	33	13	40	100
Obstruction of renal tubules	11	25	40	100
Nephritis	11	0	20	88
Decreased splenic lymphoid tissue	22	13	40	100
Increased extra-medullary hematopoiesis	67	0	40	88
Large droplet fatty change in liver	100	100	0	0
Atrophy of liver parenchymal cells	0	0	0	100
Decreased lymphoid tissue in mesenteric nodes	22	13	60	100
Atrophy of glandular mucosa of stomach	11	13	0	50
Sub-acute inflammation of stomach	11	25	40	16
Proliferation of squamous mucosa of stomach	11	13	40	50
Sub-acute inflammation of jejunum	0	0	0	50
Hemosiderosis of mucosal stroma of cecum	0	0	0	86
Calcification in aorta, coronary arteries or myocardium	0	13	0	50

[1] The figures in parentheses denote the numbers of animals sacrificed per group.

(67%) of apparently increased splenic extramedullary hematopoiesis, and for both lard and the cottonseed oil mono- and diglycerides there was a 100% incidence of large droplet fatty change in the liver. The latter finding in the controls, however, is not unexpected for this high level (25%) of fat ingestion. PMS No. 1 caused a 40% incidence of renal calculi, of obstruction of the renal tubules, of markedly decreased splenic lymphoid tissue, of apparently increased extramedullary hematopoiesis, of subacute inflammation of the stomach, and of proliferation of the squamous mucosa of the stomach, and a 60% incidence of decreased lymphoid tissue in the mesenteric lymph nodes. Microscopically in rats, PSML showed the greatest amount of involvement (as well as the highest degree of severity) in fourteen of the sixteen outstanding microscopic observations listed, of which the following were most prominent: atrophy of the testicular tubules (100%), calcified concretions in the kidney (100%), obstruction of the renal tubules (100%), decreased splenic lymphoid tissue (100%), atrophy of the liver parenchymal cells (100%), decreased lymphoid tissue in the mesenteric nodes (100%), nephritis (88%), increased splenic extramedullary hematopoiesis (88%), decreased spermatogenesis (86%), and hemosiderosis of the mucosal stroma of the cecum (86%).

In Table 7 are presented the per cent incidence of the most significant histopathological findings in hamsters fed lard, lard mono- and diglycerides, PMS No. 1, PMS No. 2, and PSML at the 5, 10, and 15 per cent levels. Such minimal findings as the presence of some fine granules of hemosiderin in rare hepatic cells or a few casts in

TABLE 7

Summary of incidence of outstanding histopathological findings on hamsters fed at 5, 10, and 15% levels for 28-39 weeks (values are per cent of animals affected).

Dietary variable		Liver		Kidney			Spleen		Stomach		Cecum	Testis	Bladder
	No. hamsters sacrificed[1]	Hemosiderosis	Cirrhosis	Obstructive nephropathy	Pyelonephritis	Atrophy	Hemosiderosis	Atrophy of follicles	Inflammation—atrophic and non-specific	Ulceration	Hemosiderosis	Atrophy	Inflammation
		%	%	%	%	%	%	%	%	%	%	%	%
5% lard	(10)	10	0	0	0	0	20	0	10	0	0	10	0
10% lard	(9)	0	0	0	0	0	0	0	33	0	0	11	0
15% lard	(9)	0	0	0	0	0	11	0	0	0	0	11	11
All 3 levels		4	0	0	0	0	11	0	14	0	0	11	4
5% LMDG[2]	(10)	0	0	0	0	0	20	20	20	10	20	10	10
10% LMDG	(10)	0	0	0	0	0	20	0	10	0	0	30	10
15% LMDG	(10)	0	0	0	0	0	10	0	20	0	0	10	0
All 3 levels		0	0	0	0	0	17	7	17	3	7	17	7
5% PMS No. 1	(7)	85	42	71	14	28	85	0	57	28	100	14	0
10% PMS No. 1	(10)	90	60	60	40	30	70	20	40	10	100	20	60
15% PMS No.1	(5)	100	40	40	20	20	100	40	40	20	100	60	40
All 3 levels		91	50	59	27	27	82	18	45	18	100	27	36
5% PMS No. 2	(8)	88	25	37	25	0	75	13	25	0	88	25	13
10% PMS No. 2	(7)	57	14	85	28	42	85	42	28	14	100	28	14
15% PMS No. 2	(4)	100	75	0	25	0	75	25	0	0	100	0	25
All 3 levels		79	32	47	26	16	79	26	21	5	95	21	16
5% PSML	(9)	78	44	67	44	33	78	11	33	0	89	44	11
10% PSML	(2)	100	50	50	0	0	100	50	50	0	100	50	0
15% PSML	(1)	100	100	100	100	0	100	100	0	0	100	100	0
All 3 levels		83	50	67	42	25	83	25	33	0	92	50	8

[1] The original number of hamsters in each group was 10, and the difference represents those that died during the experiment.
[2] LMDG = Lard mono- and diglycerides.

isolated collecting tubules, etc., which cannot be considered significant departures from normal were not recorded in the tabulation. The degrees of severity over and above these minimal deviations were reported as slight, moderate, marked or very marked. The control hamsters fed lard or lard mono- and diglycerides showed no unusually high incidence or degree of severity of microscopic findings. For the lard-fed hamsters the highest incidence was 3/28 of hemosiderosis of the spleen (3 slight); and 4/28 of gastritis (3 slight, 1 moderate). Of the control hamsters fed lard mono- and diglycerides 5/30 had hemosiderosis of the spleen (4 slight, 1 moderate); 5/30 showed atrophy of the testis (4 slight, 1 marked); and 5/30 exhibited gastritis (4 slight, 1 marked).

PMS No. 1 caused an incidence of 20/22 of hemosiderosis of the liver (3 slight, 6 moderate, 6 marked, and 5 very marked); 11/22 of hepatic cirrhosis (7 slight, 3 moderate, 1 marked); 13/22 of obstructive nephropathy (7 slight, 3 moderate, 2 marked, and 1 very marked); 6/22 of pyelonephritis (6 slight); 6/22 of atrophy of the kidney (1 slight, 3 moderate, 2 marked); 18/22 of hemosiderosis of the spleen (8 slight, 6 moderate, 3 marked, 1 very marked); 4/22 of atrophy of the splenic follicles (3 slight, 1 marked); 9/22 of gastritis (6 slight, 3 moderate); 4/22 of ulceration of the stomach (4 slight); 22/22 of hemosiderosis of the cecum (2 slight, 11 moderate, 6 marked, 3 very marked); 6/22 of atrophy of the testis (4 moderate, 2 very marked); and 8/22 of cystitis (5 slight, 1 moderate, 2 marked).

The incidence and degree of severity of the various outstanding microscopic observations in hamsters fed PMS No. 2 were 15/19 for hemosiderosis of the liver (2 slight, 3 moderate, 7 marked, 3 very marked); 6/19 for cirrhosis of the liver (3 slight, 3 moderate); 9/19 for obstructive nephropathy (5 slight, 1 moderate, 3 marked); 5/19 for pyelonephritis (4 slight, 1 marked); 3/19 for atrophy of the kidney (2 moderate, 1 marked); 15/19 for hemosiderosis of the spleen (8 slight, 6 moderate, 1 marked); 5/19 for splenic atrophy (1 slight, 3 moderate, 1 marked); 4/19 for gastritis (3 slight, 1 moderate); 18/19 for hemosiderosis of the cecum (1 slight, 7 moderate, 9 marked, 1 very marked); 4/19 for atrophy of the testis (1 moderate, 3 marked); and 3/19 for cystitis (2 slight, 1 moderate).

Of the 12 surviving hamsters (from the original 30) fed PSML, 10/12 showed hemosiderosis of the liver (2 moderate, 6 marked, 2 very marked); 6/12 had hepatic cirrhosis (4 slight, 1 moderate, 1 marked); 8/12 exhibited obstructive nephropathy (2 slight, 4 moderate, 2 marked); 5/12 had pyelonephritis (2 slight, 2 moderate, 1 very marked); 3/12 had atrophic kidneys (1 slight, 2 moderate); 10/12 developed hemosiderosis of the spleen (3 slight, 5 moderate, 1 marked, 1 very marked); 3/12 showed atrophy of the splenic follicles (1 slight, 1 moderate, 1 marked); 4/12 manifested gastritis (1 slight, 3 moderate); 11/12 presented hemosiderosis of the cecum (1 slight, 3 moderate, 4 marked, 3 very marked); and 6/12 showed testicular atrophy (2 slight, 3 marked, 1 very marked).

DISCUSSION

The oral LD_{50} values in the hamster for single doses of PMS No. 1 and PMS No. 2 were, respectively, 19.5 and 27.0 ml./kg. On the basis of 8 repeated daily doses in the hamster LD_{50} values of 1.36 and 1.44 ml./hamster/day, respectively, were obtained, equivalent to 16.2 and 16.9 ml./kg./day. The order of increasing acute oral toxicity in the hamster was found to be PMS No. 2 < PMS No. 1 < PSML. In the rat the following order of increasing acute oral toxicity was obtained: PMS No. 2 < polyoxyethylene-4-sorbitan monostearate < polyoxyethylene-20-sorbitan monooleate < glyceryl monolaurate < PMS No. 1 < PSML < sorbitan monostearate < polyoxyethylene portion of PMS No. 2. No LD_{50} values could be obtained for the following control materials in the rat despite inordinately high doses (90 ml./kg.), sufficient to distend the stomach and cause overflowing into the duodenum: cottonseed oil monoglyceride, cottonseed oil mono- and diglycerides, cottonseed oil, lard mono- and diglycerides, prime steam lard and distilled water.

The ability of the polyoxyethylene monostearates (PMS No. 1 and PMS No. 2) to convert the alkaline, cloudy, precipitate-laden urine of the normal rabbit to a clear, highly acid, precipitate-free urine containing 5.9 to 6.5 mg. polyoxyethylene glycol per ml. is of interest and should be given further study.

Of the 30 rats fed 25% levels of PMS No. 1, PSML, lard and cottonseed oil mono- and diglycerides, and sacrificed after 21 weeks, none showed complication by the usual "spantaneous" rat diseases. There was complete absence of pneumonitis, and no evidence of bartonella, paratyphoid, or any of the parasitic diseases which sometimes complicate the histological picture in a long-term study. The total of 150 hamsters fed 5, 10, and 15% levels of PMS No. 1, PMS No. 2, and PSML, with lard and lard mono- and diglycerides at the same levels as the control materials, likewise showed no evidence of complication by the "usual diseases."

The rats fed 25% levels of PMS No. 1 and PSML showed considerable evidence of irritation of the gastro-intestinal tract—markedly distended ceca (in some cases as much as five times normal size) containing large volumes of foul, fluid material; ulceration of the tail at the region of the anus with acute inflammation and abundant granulation; chronic diarrhea; abnormal presence of inflammatory cells in the stomach, jejunum, and cecum; and increased hemosiderin in the mucosal stroma of the cecum. The hamsters fed 5, 10, and 15% levels of PMS No. 1, PMS No. 2 or PSML manifested the following signs of gastro-intestinal irritation: moderate to severe diarrhea in each one of the 90 experimental animals during the course of the experiment, enlargement of the cecum, gastritis, jejunitis, cecitis, and hemosiderosis of the cecum. The inflammation noted in the various parts of the gastro-intestinal tracts of the experimental hamsters was usually of a sub-acute variety with plasma cell and eosinophile infiltration. The simplification of the architecture and the decreases in the number of the crypts of Lieberkühn likewise indicated irritation. The gastritis was most frequently seen in the antral portion of the stomach. There was no consistent tendency for the cecum to be highly inflamed even when it showed very marked hemosiderosis. On the other hand, there was only rare evidence of diarrhea (0/18 for rats; 2/60 for hamsters) and no significant observations of gastro-intestinal involvement in the control rats fed lard or cottonseed oil mono- and diglycerides at the 25% level and in the control hamsters fed lard or lard mono- and diglycerides at the 5, 10, or 15% levels.

The distribution of the unusually high number of instances of hemorrhage (678) from the genito-urinary tract in these hamsters, reported in detail as to severity elsewhere *(6)*, was as follows: 3% of the total instances of hemorrhage occurred in 9 of the lard-fed hamsters; 5% of the total involved 7 of the hamsters fed lard mono- and diglycerides; 44% of all instances of hemorrhage involved 25 of the PMS No. 1-fed animals; 40% of the hemorrhages came from 22 of the hamsters fed PMS No. 2; and 7% of the genito-urinary hemorrhages involved 8 of the hamsters fed PSML. In the case of the latter, however, it should be pointed out that of the original 30 hamsters fed PSML, 11 of the 18 fatalities had occurred within the first 7 weeks of the experiment, before any symptoms of bleeding had

been noted in any of the 71 hamsters subsequently involved. With few exceptions the degree of hemosiderosis particularly in the liver and cecum was far greater in those animals which had a previous history of bleeding from the genito-urinary tract than in the hamsters which did not manifest this finding. This may, however, be coincidental. There was no correlation between the number of instances or the degree of severity of these hemorrhages and the per cent cellularity, the degree of maturity or the distribution of myeloid and erythroid elements of the bone marrow, or the degree of obstructive nephropathy. The episodic nature of these hemorrhages which varied from a single instance to repeated hemorrhages lasting for as much as 15 days, followed by days, weeks, or even months before the next bleeding occurred, and the high incidence of microscopic evidence of marked dilatation and obstruction of the convoluted and collecting tubules with hyaline and calcific material suggest the possibility of movement of concretions as the etiological factor.

The consistently high incidence and marked degree of severity of the following histopathological findings after feeding polyoxyethylene derivatives (PSML, PMS No. 1, and PMS No. 2) to rats at 25% levels for 21 weeks and to hamsters at 5, 10, and 15% levels for 28 to 39 weeks are most significant: atrophy of the testes with decreased spermatogenesis, renal atrophy, nephritis, obstructive nephropathy, cirrhosis and hemosiderosis of the liver, decreased splenic lymphoid tissue, hemosiderosis of the spleen and hemosiderosis of the cecum. The most outstanding microscopic changes observed in the hamsters fed these agents were the striking frequency and severity of deposition of yellow-brown pigment resembling hemosiderin chiefly in the parenchymal cells, lymphatics and Kupffer cells of the liver, in the follicles and red pulp of the spleen, and in the cecum. Tests on some of these tissues with Gmelin's reagent gave negative results; this finding and the complete absence of pigment in the bile ducts definitely ruled out bile pigment. Positive tests for iron with the Prussian blue reaction (employing potassium ferrocyanide and hydrochloric acid) added further confirmation of the identity of the pigment as hemosiderin. That the pigment did not accumulate because of hemorrhage in these particular organs is evident from its diffuse nature and the absence of marked, inflammatory changes. Since there was no evidence of previous or recent injury to the liver cord cells or the epithelium of the cecum, particularly in those cases where the deposition of pigment was very slight, it would seem unlikely that this exaggerated pigment deposition resulted from hepatic necrosis or ulceration of the cecum. This leaves three other possibilities in explanation of the very marked and widespread hemosiderosis: (a) overabsorption of iron because of marked surface activity in the gastro-intestinal tract and failure to utilize the excess, (b) generalized blood destruction, or (c) local hemolysis. This might prove to be an interesting experimental and clinical tool for the production and study of hemosiderosis and merits further investigation. Wissler *et al. (10)* have already reported experiments with Fe^{59} in hamsters fed polyoxyethylene sorbitan monolaurate in which there was evidence of increased iron absorption accompanied by the presence of iron pigment in the cecal mucosa, mesenteric lymph nodes, and liver.

SUMMARY

Acute oral toxicity studies were performed in rabbits, hamsters, and/or rats on polyoxyethylene monostearates, polyoxyethylene-20-sorbitan monolaurate, glyceryl monolaurate, polyoxyethylene-4-sorbitan monostearate, polyoxyethylene-20-sorbitan monooleate, and sorbitan monostearate, with cottonseed oil monoglyceride, cottonseed oil mono- and diglycerides, cottonseed oil, lard mono- and diglycerides, lard and distilled water as the controls.

Repeated daily doses of two polyoxyethylene monostearates were administered orally to hamsters for eight days, and the respective LD_{50} values were 1.36 and 1.44 ml./hamster/day.

Single intubations (20 ml.) of the two polyoxyethylene monostearates in rabbits caused the normally cloudy, alkaline urine of the rabbit to become acid, clear, and free from precipitate, and to contain 5.85 and 6.5 mg., respectively, of polyoxyethylene glycol/ml.

Gross and histopathologic studies were carried out on rats which had been fed 25% levels of polyoxyethylene monostearate, polyoxyethylene-20-sorbitan monolaurate and the control substances lard and cottonseed oil mono- and diglycerides and sacrificed after 21 weeks. The most outstanding gross findings were in the rats fed polyoxyethylene-20-sorbitan monolaurate which showed 100% incidence of enlarged kidneys, renal calculi, diarrhea, and ulceration at the base of the tail, and 75% incidence of stones in the urinary bladder and of marked enlargement of the cecum. Microscopically, these same rats showed 100% incidence of the following: atrophy of the testicular tubules, calcified concretions in the kidney, obstruction of the renal tubules, decreased splenic lymphoid tissue, atrophy of liver parenchymal cells, and decreased lymphoid tissue in the mesenteric nodes. There was also high incidence of decreased spermatogenesis (86%), nephritis (88%), increased extramedullary hematopoiesis (88%), and hemosiderosis of the mucosal stroma of the cecum (86%), as well as other findings.

Gross and histopathologic studies were made on hamsters which had been fed 5, 10, and 15% levels of two polyoxyethylene monostearates and of polyoxyethylene-20-sorbitan monolaurate and sacrificed after 28-39 weeks. For each of these three compounds there was unusually high incidence (25-100%) of fatalities, chronic diarrhea, small testes, thickened bladder walls, distorted, abnormal kidneys, and enlarged ceca, and very striking hemosiderosis of the liver, cecum and spleen, cirrhosis of the liver, obstructive nephropathy, as well as many other highly significant effects. The control hamsters fed similar levels of lard or lard mono- and diglycerides showed no unusual incidence of these gross and histopathological findings.

Six large stones 4.5-5.5 mm. in diameter and varying from 53-94 mg. in weight were removed from the urinary bladders of hamsters which had been fed polyoxyethylene monostearates for periods varying from 74 to 260 days.

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THE B VITAMIN CONTENT OF GRAPES, MUSTS, AND WINES

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Vitamin content of grapes, musts, and wines has been examined frequently but the losses or gains produced by the processes of crushing and fermentation have not been determined systematically. A study of the thiamine, riboflavin, vitamin B_6, and pantothenic acid (filtrate factors) of several California grown grape juices and wines was reported in 1939 *(14)* and of the stability of added portions of these four vitamins in similar preparations in 1945 *(15)*. Castor *(6)* noted the changes in B vitamin content of fermenting grapes musts and wines with special reference to the effect of the yeast action and of aging. A parallel study of the whole grapes and their musts has not been reported. The literature on vitamins in wines has recently *(1)* been reviewed.

In this experiment five varieties of grapes of the vintages of 1952, 1953, and 1954 were used, the juices expressed in various ways and the wines produced under varying conditions. Two varieties of white and three of red grapes were employed and the wines made from them were of the dry white, sherry, sweet red, dry red, and dry rosé types. A description of the products is given in Table 1.

EXPERIMENTAL PROCEDURE

During September and October 1952, 1953, and 1954 samples of fresh grapes and freshly pressed musts were prepared and stored at once in the freezing room (−10°C.). The grapes were sampled both as the whole fruit and as a slurry after the stems had been removed. Samples of the wines made from the musts were also stored a few days after fermentation when the yeast had settled and the supernatant liquid could be removed. Finished wines, aged in the usual cellar fashion, and stored at 53°F., were examined 4 to 6 months later. The musts were fermented with a pure yeast culture (Montrachet strain of *Saccharomyces cerevisiae* var. *ellipsoideus)* and in all cases except one, 75 p.p.m. SO_2 were added before fermentation. In one sample of White Riesling wine no sulfiting was used and in another 125 p.p.m. SO_2 were added before fermentation and 100 p.p.m. 6 weeks later. The red wines were produced by fermentation of the crushed grapes, the pink and white wines by fermentation of the expressed juices.

Analytical methods. A modification of the thiochrome method was used for thiamine *(7)* and both the fluorometric procedure *(12)* and the usual microbiological method by use of *L. casei* were applied for riboflavin. Results of the fluorometric method agreed fairly with those of the microbiological only in the musts and wines. Much larger values were obtained by the former with all the grape samples. Obviously non-riboflavin fluorometric substances were present in considerable amounts in the skins and seeds, thus invalidating the method. In general the values obtained by fluorometry were larger even for the musts and wines than were those obtained by the usual microbiological procedure. In consequence only the values from the microbiological tests are used in the summaries and comparisons.

Niacin was determined by the microbiological method of Snell and Wright *(19)* and pantothenic acid by the similar method of Skeggs and Wright *(17)*. The extraction for pantothenic acid was done with mylase-P by the method of Ives and Strong *(9)*.

TABLE 1

Composition of musts and wines

Year	Grapes	Musts			Wines					
		Total acid % tartaric	Balling degrees	pH	Ethanol % vol.	Extract %	Reducing sugar	Total acid % tartaric	pH	Tannin %
1952	White Riesling	0.87	21.0	3.33	11.1[1]	2.6	0.04	0.77	3.16	0.03
					11.2[2]	2.5	0.04	0.80	3.12	0.03
	Palomino	0.53	18.7	3.65	19.5[3]	4.7	0.19	0.36	3.71	0.03
	Tinta Madeira	0.82	22.1	3.48	12.5[4]	2.6	0.04	0.73	3.60	0.03
					11.5[5]	3.3	0.05	0.65	4.08	0.11
					19.8[6]	12.6	8.8	0.60	4.25	0.07
	Xeres	0.66	22.3	3.60	19.5[6]	11.5	6.5	0.36	3.89	0.05
	Zinfandel	0.88	15.5	3.45	8.4[5]	2.7	0.07	0.60	3.45	0.04
1953	Palomino	0.54	17.8	3.50	10.0[2]	2.3	0.08	0.65	3.22	0.01
					19.3[3]	2.3	0.34	0.46	3.42	0.02
	White Riesling	0.70	21.5	3.49	11.8[2]	2.8	0.16	0.77	3.12	0.02
	Tinta Madeira	0.86	22.7	3.70	19.5[6]	14.2	11.0	0.81	4.12	0.05
	Xeres	0.83	20.9	3.40	18.6[6]	13.8	11.6	0.67	3.69	0.06
	Zinfandel	1.00	22.6	3.30	11.4[5]	3.6	0.19	1.02	3.34	0.11
1954	White Riesling	0.72	22.5	3.39	13.0[2]	2.5	0.10	0.55	3.10	0.02
	Tinta Madeira	0.83	24.5	3.48	13.5[4]	2.4	0.15	0.72	3.30	0.01
		0.80	24.6	3.40	12.4[5]	2.7	0.14	0.55	3.90	0.09
		0.80	25.0	3.40	19.2[6]	13.2	9.0	0.64	3.98	0.07

[1] Dry white wine, no SO_2
[2] Dry white wine, 125 ppm SO_2 on crushing, 100 ppm after fermentation
[3] Sherry wine, dry fortified, baked
[4] Dry rose wine, fermented liquid must
[5] Dry red wine, on skins 3 days
[6] Sweet red wine, on skins 3 days, fortified

For vitamin B_6 (pyridoxine) the method of Atkin *et al. (2)* was used, and in 2 series a rat-growth method *(8)* was also incorporated.

Freshly prepared and dried chicken pancreas and fresh and dried hog kidney enzyme extracts were used for the extraction of folic acid. The desiccated chicken pancreas preparation was selected because it contained the least folic acid, producing blanks with about 0.02 meg. folic acid. Samples were autoclaved for 5 minutes before enzyme treatment. This was as effective as the 15 minutes recommended by Bird *et al. (3)*. "Free" folic acid was determined in a number of samples with both the hog kidney and chicken pancreas enzymes and with and without initial autoclaving. Of the total folic acid of grapes, juices, and wines, from 67 to 90% was found to be "free," or determinable without enzyme extraction.

The folic acid method finally adopted involved measurement of 5 g. of grapes, or 10 ml. of juice or 15 ml. of wine into a 125-ml. colored flask with 10-20 ml. of 0.2 M KH_2PO_4 buffer, and the volume brought to 35 ml. The mixture was then autoclaved for 5 minutes at 121°C., cooled, 5 ml. (20 mg.) chicken pancreceas preparation and

1 ml. toluene aded and the whole incubated at 37°C. for 24 hours. After incubation the samples were autoclaved for 5 minutes at 121°C., cooled, made to volume and filtered. Tomato juice agar and broth made according to Lyman *et al. (13)* were used for growing the organism, which was *Streptococcus faecalis 8043* grown in Bacto dehydrated medium according to the formula of Capps *et al. (5)*. The standard was synthetic pteroylglutamic acid. Incubation was for 72 hours at 36-37°C. and the response was measured by titration with 0.1 *N* NaOH.

Vitamin B_{12} activity of most of the samples was so low as to make determination difficult. Extraction was effected by the method used in the A.O.A.C. collaborative study in 1952 *(10)* and the assay carried out by use of *L. leichmannii 7830* using Difco dehydrated medium B457.[a] Considerable difficulty resulted from the inhibition of growth of the organism caused by the invert sugar content of most of the samples. Many devices were tried to avert this inhibition, such as digestion with pectinol, trypsin, takadiastase, and papain, or pancreatin. The B_{12} content of the enzymes was found in all cases to be greater than that of the samples and the inhibition was not lessened. Treatment with alcohol, acetone or ether was ineffective. The KCN conversion of B_{12} to cyanocobalamin with absorption on alumina *(11)* or on charcoal *(16)* and elution with water or acetone proved useless with these low potency samples. Response curves for 5, 10, 15, and 20% glucose solutions containing 0.01 μ mcg./ml. B_{12} were obtained (Figure 1) and the 5% solution shown to give satisfactory curves. All samples were therefore diluted to 5% or lower sugar concentration before the assays.

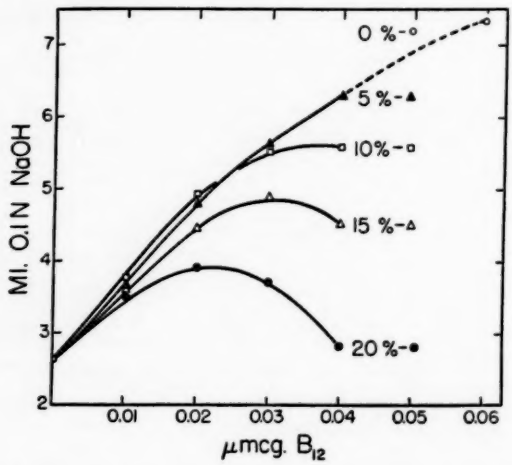

Figure 1. Effect of (%) glucose concentration on apparent vitamin B_{12} activity as shown by growth of L. leichmannii.

The interference of bisulfite with the growth of the organism was tested since nearly all the wines contained bisulfite and bisulfite is used in B_{12} extraction. Dilution of the extracts was not practical since the B_{12} activity was already very low. Limits of bisulfite concentration for non-interference with the growth of *L. leichmannii* were examined. Each concentration was found to present a characteristic effect upon the growth of the organism. Thus it was necessary to ensure similar amounts of bisulfite and of vitamin B_{12} in samples and standards. After preliminary tests, for example, Zinfandel and Xeres musts which showed significant B_{12} activity were extracted with 0.03% and 0.06% bisulfite respectively and compared with standards of similar bisulfite content.

[a] Prepared according to the formula in Third Suppl. USP XIV, p. 15, 1951.

RESULTS

There was measurable *vitamin B_{12}-like activity* in both white and red grapes, juices and wines, about twice as much usually in the red as in the white, 1.2 and 2.5 μmcg. per 100 g. or ml., respectively, in the grapes and 0.9 and 2.3 in the musts. The sweet red and dry red dessert wines appeared to contain 1.5 to 2.8 μmcg. vitamin B_{12} per 100 ml., the dry white and dry pink wines 0.3 to 1.0. In 1953 Castor *(7)* found no activity in the must but development up to 2.5 μmcg. per 100 ml. after 36 hours of yeast fermentation then a slow decline. Values reported by Peynaud and Lafourcade *(16)* were larger, 0 to 13 for musts, and 0 to 16 for wines. The organism used was *Escherichia coli* 113-3. Some loss generally occurred during fermentation of the musts as measured in our experiments but little or none during expression of the musts. The significance of this slight vitamin B_{12}-like activity in wine grapes and their products is difficult to assess. Some other substances such as pyrimidines or nucleotides may account for the stimulus to the growth of *L. leichmannii 7830*.

The *folic acid* values are shown along with those of the other B vitamins in Table 2. The red grapes again had higher values, 5.8 to 10.2 mcg. per 100 g., than the white, 4.2 to 5.3. A loss of 46 to 78% of this activity occurred in the extraction of the musts but there was little further loss or some increase in fermentation to wine. The baked Palomino wine (sherry material) had less folic acid than the unbaked and the sulfited White Riesling wine less than the unsulfited. The sweet red wines, from Tinta Madeira and Xeres grapes, had significantly higher folic acid activity than the dry red or dry white wines. The skins and seeds of the grapes may have contributed folic acid as well as other B vitamins to the finished wines. The folic acid values found for these wine grapes are in line with those given for grapes and raisins by Toepfer *et al. (20)*.

The *thiamine* contents of the 1953 and some of the 1954 samples were found to resemble those reported earlier *(14, 15)*. There was little variation among the grapes since all showed about 40 mcg. per 100 g. The musts contained nearly as much, losses in extraction varying from 3 to 23%. During fermentation with 75 p.p.m. SO_2 however most of the vitamin was destroyed. The sweet red wines retained a measurable amount as did the dry rosé Tinta Madeira wine in 1954. These musts and wines had thiamine contents very similar to those reported in 1939 *(14)* and 1945 *(15)* and by Cailleau and Chevillard *(4)*. It should be noted that most of the determinations in the earlier California studies were made by rat growth rather than the thiochrome method used in the present study. Rat growth vitamin assays usually yield higher values than do chemical or microbiological methods.

Not only the sulfiting usually involved but the yeast growth itself may contribute to the loss of thiamine in the wines.

Riboflavin has been assayed in grapes, juices, and wines more frequently than any other vitamin. It was shown *(15)* to be unusually light-labile and to be protected effectively only when dark glass was used for bottling. The amount found in grapes in this study was remarkably uniform, 20 to 27 mcg. per 100 g. The musts contained one-half to two-thirds these amounts and the wines retained nearly all of those in the musts. The red wines generally retained more of the must riboflavin than did the white. It has been shown *(6, 14)* that riboflavin concentration may be increased during fermentation but in storage, especially in the light, this is lowered. Perhaps the dark color of the red wines is protective against the action of the light *(4)*.

Niacin was found in all the samples in much higher concentration than any of the other vitamins. Here again the red grapes and their products were better endowed than the white. The musts contained 70 to 80% of the grape niacin and the wines widely varying proportions. The white wines contained 33 to 56% of the must niacin, the dry red wines 30 to 100, the dry pink wine 20, and the sweet red wines 90 to 114. Castor *(6)* noted a similar range of occurrence as did Cailleau and Chevillard *(4)*.

The *vitamin B_6* group next to niacin was most concentrated in these grapes, 69 to 163 mcg. per 100 g. Fifty to 90% of the grape pyridoxine was retained in the musts and about 40% of this remained in the white wines, 60% or more in the red wines. Two rat growth assays for vitamin B_6 were also made with the 1952 Xeres and Tinta Madeira grapes, musts and sweet red wines. The method has been described *(8)*. In the Tinta Madeira bioassay, ethanol was added to the 6 mcg. pyridoxine daily dose fed as standard and also to the must dose. This was the amount present in the wine daily dose. The gains made in 4 weeks by the rat groups given 6 mcg. pyridoxine with and

TABLE 2

B Vitamin content of wine grapes, musts and wines in mcg./100 gm. or ml.[1]

Year and Variety	Product	Moisture %	Thiamine	Riboflavin		Niacin	Vitamin B6	Pantothenic acid	Folic acid
				Microbiological	Fluorometric				
Palomino	grapes	79.8					84	79	4.2
1952	must	82.3					66	64	1.4
	dry wine						19	33	1.8
	baked sherry						17	12	1.0
1953	grapes	81.5	38	21	27	172	88	49	5.1
	must	82.4	29	10	12	155	65	45	1.6
	dry wine		3	6	13	59	23	55	1.9
	baked sherry		5	11	5	88	20	....	0.8
White Riesling	grapes	75.0					91	77	5.3
1952	must	81.1					74	63	1.1
	dry wine + SO_2						25	33	1.2
	dry wine no SO_2						27	32	1.6
1953	grapes	75.2	39	20	74	237	87	68	5.1
	must	77.9	38	12	18	219	65	80	3.2
	dry wine + SO_2		1				35	63	2.4
1954	grapes	73.1	47	24	86	252			
	must	77.9	36	9	14	165			
	wine		1	11	9	55			
Tinta Madeira	grapes	75.6					126, 105[3]	88	5.8
1952	must	78.7					63, 62[2]	74	1.2
	sweet red wine						54, 48[2]	19	3.4
	dry red wine						53	13	1.3
	dry rosé wine						46	20	3.2
1953	grapes	74.5	51	23	93	318	110	84	6.5
	must	77.8	65	16	12	214	60	74	1.7
	sweet red wine		18	24	41	242	61	36	3.1

(Table 2 continued on next page)

TABLE 2 (Concluded)

Year and Variety	Product	Moisture %	Thiamine	Riboflavin		Niacin	Vitamin B6	Pantothenic acid	Folic acid
				Microbiological	Fluorometric				
1954	grapes	71.4	58	24	133	329	113		
	must	75.6	51	13	15	206	73	65	1.7
	must rosé	76.5	51	13	15	213	79	65	1.8
	sweet red wine		16	27	34	190	57	22	2.5
	dry red wine		0	18	24	65	34	30	2.1
	dry rosé wine		1	13	14	41	27	37	1.1
Xeres	grapes	73.2					163[2], 118	107	9.4
1952	must	76.2					101[2], 106	84	1.4
	sweet red wine						66[2], 84	35	2.6
1953	grapes	75.7	50	25	93	275	126	82	8.2
	must	80.9	44	12	18	157	72	81	1.6
	sweet red wine		6	20	26	184	66	30	2.4
Zinfandel	grapes	77.1					113	69	7.1
1952	must	83.3					100	62	2.3
	dry red wine						39	46	1.7
1953	grapes	72.0	38	22	120	315	135	81	10.2
	must	78.9	31	9	20	245	101	100	3.9
	dry red wine		1	7	27	127	54	59	1.3

[1] Each figure represents 2 to 6 assays
[2] By rat bioassay

without 7.3 ml. 20% alcohol daily were almost identical, 86 and 87 g. The same was true of the must fed with and without same amount of alcohol. Evidently the presence of this concentration of alcohol, about 20% in the wine doses, did not interfere with the absorption and utilization of its vitamin B_6. The vitamin B_6 activity of the must and wine samples agreed well when determined by the microbiological and biological methods. The Xeres grape samples showed about 38% more and the Tinta Madeira grapes about 20% less vitamin B_6 value by rat growth than by the yeast growth method. These differences may be due to differences in sampling or in digestibility of the grapes. Both musts and wines might be expected to be free from such variability and this is indicated by the closer agreement of the values found by the two methods.

Pantothenic acid concentration was similar in magnitude to that of vitamin B_6 but slightly lower and fairly uniform. Sixty-eight to 93% of the grape content was

found in the musts and one-fourth to one-half of the must content in the wines. In most cases the dry wines, both white and red, retained this vitamin better than did the sweet dessert wines. All of the wine values are lower than those reported by Cailleau and Chevillard *(4)* but are similar to those of Castor *(6)*.

Average values of the concentrations of six B-vitamins in these grapes, musts, and wines, red and white varieties separately, are shown in Table 3, and the losses in must production and vinification in Table 4.

TABLE 3

Average vitamin contents of red and white grapes, musts, and wines

Vitamin	No. of lots	Grapes		Musts	Wines				
		Variety	mcg/ 100 gm	mcg/ 100 ml	Dry white mcg/ 100 ml	Dry white dessert mcg/ 100 ml	Dry rose mcg/ 100 ml	Dry red mcg/ 100 ml	Sweet red dessert mcg/ 100 ml
Thiamine	3	White	41	34	2	5			
	4	Red	49	50			1	1	13
Riboflavin	3	White	22	10	9	11			
	4	Red	24	14			13	13	24
Niacin	3	White	220	180	57	88			
	4	Red	309	207			41	96	205
Vitamin B6	4	White	88	67	26	19			
	6	Red	125	83			37	47	64
Pantothenic acid	4	White	68	63	37	12			
	6	Red	85	76			29	37	24
Folic acid	4	White	4.9	1.8	1.8	0.9			
	6	Red	7.9	2.0			2.1	1.5	2.9

DISCUSSION

Changes in these constituents caused by expression of the grapes and fermentation of the musts are shown in Figure 2. It is evident that thiamine and pantothenic acid suffer little loss in the expression of the juice from

TABLE 4

Losses of vitamins in juice extraction and vinification of white and red wine grapes

Vitamins	% loss in						
	Must production		Vinification of must				
	White	Red	Dry white	Sherry	Dry rosé	Dry red	Sweet red
Thiamine	17	0	94	86	98	98	74
Riboflavin	54	42	30	0	7	7	+71
Niacin	18	33	69	51	80	54	1
Vitamin B_6	24	33	60	72	55	43	24
Pantothenic acid	8	10	41	80	62	51	68
Folic acid	63	75	0	50	+5	25	+45

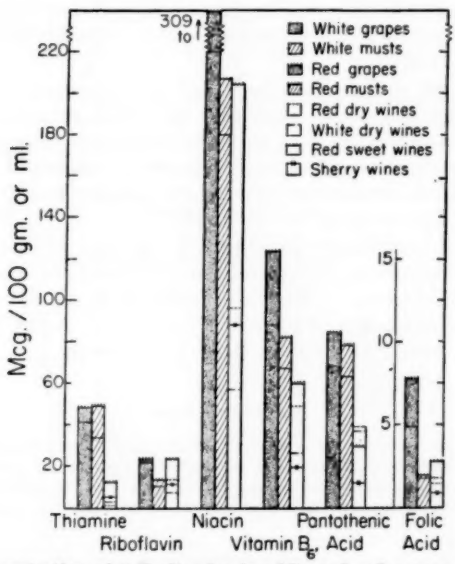

Figure 2. Concentration of 6 B vitamins in white and red grapes and musts and in the 4 types of wines made from them.

the grapes but considerable destruction in the fermentation process. Thiamine in fact is nearly completely destroyed especially in the sulfited wines but 20 to 50% of the pantothenic acid of the musts appear in the wines. Riboflavin and folic acid on the other hand suffer the main losses in juice extraction, and little or no change, or in the sweet red wines an increase, in fermentation. The light lability of riboflavin and folic acid may account for these changes and if the wines are bottled in clear glass during commercial handling these vitamins may deteriorate. All the samples examined in this study were kept at all times in dark cellars or in a dark freezing room. The contribution of skins and seeds of the grapes may also help to account for the higher values of the red wines. These analyses may indicate little contribution of thiamine and pantothenic acid by the grape skins but much riboflavin and folic acid. The niacin and vitamin B_6 of the grapes were lost to the extent of one-third or less in must production and one-half or more of this residue in fermentation. A curious exception was found again in the sweet red dessert wines, which retained most of the must values of these vitamins. The superiority of the sweet wines in vitamin retention may be in part due to their lesser amount of fermentation as shown by their retention of sugar. The traditional choice of port type wines for blood building may have some justification.

SUMMARY

Thiamine, riboflavin, niacin, vitamin B_6 (pyridoxine), pantothenic acid, folic acid, and vitamin B_{12}-like activity were determined in two varieties of white wine grapes, their musts, and dry table or dessert white wines

and in three varieties of red wine grapes, their musts and dry red table, dry rosé table, and sweet red dessert wines. Samples of three different vintages were examined.

The red grapes and musts were richer in all of these vitamins than the corresponding white grapes and musts. The same superiority was seen in the red wines except in the case of pantothenic acid. The wines fermented on the skins, dry red, were not much superior to those not so treated, rosé dry, but the red sweet dessert wines were superior to all the other wines in retention of the vitamins of the must.

The thiamine and pantothenic acid of the grapes disappeared chiefly in the vinification process, but riboflavin and folic acid disappeared in the extraction of the musts. Niacin and vitamin B_6 decreased during both processes, more obviously in fermentation, except in the sweet red wines which retained nearly all of these must constituents.

Acknowledgments

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PAPER DISK METHOD FOR SUBCULTURING ACIDIFIED FOOD PRODUCTS[a]

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(Manuscript received November 25, 1955)

During an investigation on the inhibitory and preservative action of various acetic acid and soluble solids concentrations in different foods towards acid food spoilage microorganisms, it was necessary to employ a confirmatory recovery technique to determine the presence or absence of viable organisms.

Cloudiness in the confirmatory media was a frequent problem in the bacteriological studies of food products. This was caused by the addition of such acidified foods as puréed corn, French dressing, mayonnaise, and puréed pickled herring. In the culturing procedure, food samples were transferred to tubes of sterile broth. A persistent cloudiness and sediment obscured evidence of growth. The problem of cloudiness was solved by the application of a modified auxanographic method. This technique incorporates the use of sterile filter paper disks, which are dipped into the confirmatory broths and aseptically transferred to the surface of sterile agar plates. Viability of the culture is indicated when growth encircles the disk.

The auxanographic technique was first used by Beijerinck *(1)* to study the nutritional requirements of microorganisms. Pontecorvo *(6)*, deBeer *(2)*, and others have used the method for metabolic studies of microorganisms. The method consisted of seeding nutritionally deficient agar plates with a test organism. Small amounts of growth factors were placed on the surface of the agar in crystals or solutions, and the requirement of the test organism was identified by a growth response. An additional variation of the method was to soak small disks of sterile filter paper in the desired solutions and place the disks on the surface of the seeded agar plates. This method has also been employed for the assay of enzymes (Sherwood, *8)*, and vitamins (Genghof, *3)*, and for the efficacy of antibiotics (deBeer, *2)*.

Reddish *(7)* in studies of antiseptics varied the technique by using agar plates or broth, which were used to culture filter paper disks impregnated with the test organism. Sherwood *(9)* prepared filter paper carriers of mycelial growth, which were then treated with fungicides, washed, and subcultured on sterile Saboraud's agar with no loss of the matted mycelial growth. In the field of bacterial genetics, Plough *et al. (5)* identified biochemical mutants by inoculating filter paper disks with the mutated bacterial strains. The disks were placed on minimal agar individually supplemented with growth factors. Lederberg's *(4)* contribution of the replica plating technique added speed to the detection of mutants and made possible his unique indirect selection test.

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[a] Contribution No. *1018* of the University of Massachusetts College of Agriculture Experiment Station.

The present paper describes the method of handling the disks, the direct application of disks to food products, and the usefulness of the disk technique in the subculturing of confirmatory broths.

MATERIALS AND METHODS

Agar plates of the required culture media are prepared a day preceding an experiment to allow moisture to be dissipated from the surface. With a wax pencil, the outside surface of each plate is divided into quarters with a direction arrow placed on the outer edge.

The filter paper disks, which are cut with a paper punch through two or more layers, are autoclaved and stored in covered petri plates. Dry heat may be used to evaporate excess moisture after autoclaving. The sterile disks are handled with small, curved forceps. The tips of the forceps are immersed in alcohol and flamed between each transfer. Each disk is dipped into the substrate to be tested and placed in a definite order on the sterile agar medium starting at the outside edge from a point appropriately marked with a direction arrow. The plates are incubated from one to three days and observed for growth around the edge of the disk, as illustrated in Figure 1.

Figure 1. Zones of microbial growth around paper disks on agar medium.

DISCUSSION

For an early survey of the effectiveness of the soluble solids and acid on the food substrates, a direct test was tried in which the paper disks were dipped into the food substrate and then transferred directly to the agar plates. For a number of reasons, the direct test was not entirely satisfactory. The consistency of the substrates determined the feasibility of using the technique. The mayonnaise and the herring were too thick, whereas the French dressing, although thin, deposited a ring of oil about each disk, which caused spotty growth. When the method was applied to the corn, the material adhered to the disks, and it was necessary to use the microscope to differentiate the corn particles from colonies.

The subculturing of confirmatory broths was found to be the most effective way of testing the preservative action of the soluble solids and acid on the inoculated food products. The confirmatory broths were made by adding 1-ml. amounts of food substrate to 15 ml. of nutrient broth. For subculture technique, the paper disks were dipped into the confirmatory broths and aseptically transferred to nutrient agar plates. The foods inoculated with yeast strains *Zygosaccharomyces globiformis*, and *Schizosaccharomyces octosporus* produced gas in the confirmatory broths, but at the bacteriostatic level growth activity was difficult to detect and had to be further confirmed by disking for colony observation. It was essential to subculture the *Lactobacilli lycopersici*, because they produced negligible amounts of gas in broth. The controls were likewise confirmed. Thus, the addition of the disk subculture technique to the regular confirmatory broth method proved practical for clarifying doubtful instances of growth in clouded broths.

SUMMARY

Confirmatory bacteriological tests of food products that produce a clouding effect are not easily observed for growth. This particular problem arose when bacteriological tests were being made on previously inoculated food products, such as acidified corn, French dressing, mayonnaise, and pickled herring. A modified auxanographic method was used successfully by subculturing the confirmatory broths on agar plates. This technique incorporated the use of sterile filter paper disks, which were dipped into the confirmatory broths and aseptically transferred to the surface of uninoculated nutrient agar plates. Positive broths were indicated when growth occurred around the edge of the paper disk. A direct application of the paper disk method for culturing the inoculated foods on agar was useful to a limited extent when a survey of the survival of the inocula was desired prior to the designated final six-week test.

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MEAT TENDERIZATION. II. FACTORS AFFECTING THE TENDERIZATION OF BEEF BY PAPAIN[a]

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(Manuscript received December 16, 1955)

The use of meat tenderizers containing the proteolytic enzyme papain has grown to large proportions. However, there have been no critical studies of the mechanism of meat tenderization by papain treatment, or of the factors influencing the effectiveness of papain tenderization of meat. Histological investigation could yield much valuable information concerning the mechanism of meat tenderization with papain; however, such studies are lacking.

In one of the first papers on the tenderization of meat with papain, Gottschall and Kies *(3)* observed a penetration of 2.5 mm. by soaking raw meat in papain solution for 20 minutes at 23°C. Penetration studies led them to conclude that there is no advantage in pretreatment because enzyme digestion takes place during the cooking process. Nevertheless, empirical precooking holding periods, usually of the order of 1 hour per each inch of thickness of the meat, are recommended commercially. For example, this precooking holding period has been used in the studies of Hay, Harrison, and Vail *(5)*. Because of the controversial aspect of the effect of the precooking holding period, a more critical evaluation of enzyme penetration and hydrolytic activity at room temperature and of the effect of this precooking holding period seems appropriate.

The mechanism of meat tenderization by papain at the molecular level can be described by the relative hydrolysis of the muscle-fiber protein, mainly actomyosin, and by hydrolysis of the connective tissue, mainly collagen and elastin. Studies of the hydrolysis of various beef protein fractions by papain at 60°C. have been recently reported *(6)*.

It was the purpose of this investigation to define by appropriate physical, chemical, and histological methods the effect of time of papain application prior to cooking and the effect of temperature on protein hydrolysis by papain.

MATERIALS AND METHODS

Histological examination. For histological studies of enzyme action, a *semitendinosus* muscle from U.S.D.A. *Choice* grade of beef was used. The meat was cut into ⅝ inch-thick slices by parallel cuts along the length of the muscle fibers, and adjacent discs, 1 inch in diameter, were cut from these slices with a cork borer. Some discs were left raw, some cooked in a pressure cooker (10 p.s.i. for 20 min.), and some were coated on the upper flat surface with powdered papain (S. B. Penick and Co.) before pressure cooking as above. Several pieces of meat were cut so that the ends of the muscle fibers were exposed. Papain powder was placed on this exposed surface and samples were taken initially, after 3 hr. at room temperature and after ½ hr. at room temperature and 10 min. of broiling.

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[a] Supported in part by a grant from Adolph's Foundation.

All samples were put in 10% formalin and evacuated immediately. After 2 days they were dehydrated by a dioxane schedule and ultimately embedded in paraffin. Sections were cut at 10 μ and oriented so that the plane of cut was perpendicular to the surface powdered with papain. Staining was carried out after the method given by Hanson *(4)*.

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The hemoglobin substrate was prepared as follows: 4 ml. of 2.0 M sodium hydroxide, 78 ml. of water, 36 g. of urea, and 8 ml. of 4.2×10^{-3} M hog hemoglobin were mixed and allowed to stand at room temperature for 30-60 m²n. to denature the hemoglobin. Then, 10 ml. of 1 M potassium dihydrogen phosphate, 4 g. of urea, and 2 mg. of merthiolate were added.

The substrate used for the milk-clot method *(2)* consisted of 20 g. of instant milk (Carnation) diluted to 100 ml. with 0.1 M citrate buffer. In order to determine the reaction rate, 10 ml. of the milk was mixed with a given amount of enzyme solution in a test tube, which was stoppered and immersed in a thermostated water bath. The tube was suspended horizontally and tilted back and forth periodically until the formation of a sandy precipitate throughout the tube indicated incipient clotting. The reciprocal of time to reach incipient clotting was taken as a measure of the rate of the reaction.

Activated papain (Nutritional Biochemicals Corporation) solution was prepared by adding to 1 g. of finely ground papain 50 ml. of water, 10 ml. of 0.01 M Versene, and 1 ml. of 0.125 M cysteine-hydrochloride. This solution was then diluted to 100 ml. and filtered. Several drops of toluene were added to the filtrate and it was stored at 10°C.

Papain digestion of beef proteins. Freeze-dried *biceps femoris* was finely ground to pass a No. 10 mesh in a Wiley mill and then divided into 3 lots of six 5-g. samples.

These 3 lots had total nitrogen contents of 12.2, 12.2, and 12.4%. Each 5-g. sample was rehydrated 30 min. in 20 ml. of water at room temperature. At the end of this period, samples from each of 3 lots were placed in water baths at 0°, 20°, 40°, 60°, and 80°C. After temperature equilibration, 5 ml. of 1% aqueous papain (Nutritional Biochemicals Corporation) were mixed into each sample. The controls were held at 60°C. and were treated with water instead of papain. Digestion was allowed to take place for 1 hr., and the reaction was stopped by adding 100 ml. of 0.1 N sodium hydroxide. The methods of extraction, fractionation, and measurement of the protein components which were employed here have been described previously *(6)*. During this procedure, the combined weights of collagen and elastin were also determined.

RESULTS

Penetration of papain into meat at room temperature. After 3 hours of exposure to a thick layer of papain powder, there was no histological evidence of enzymatic activity. Below the superficial layer of enzyme powder, all the muscle fibers were intact, with few noticeable disturbances in the striation pattern. Similarly, no changes were evident in the collagenous or elastic fibers when the treated meat was compared with the untreated control. A slight coalescence of muscle fibers at the outermost surface of both the treated and untreated samples may perhaps be related to a slight drying of this surface on standing.

The study of papain penetration into cubes of meat by the use of quinine sulfate and bromcresol green indicators showed penetrations ranging between 1 and 2 mm. No definite relation could be established between time, temperature, and penetration.

Effect of the precooking period on papain tenderization of beef. Results of 5 experiments (Table 1) revealed a significant difference between the

TABLE 1
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Precooking holding-period, hrs.	Averages of shear (ft. lbs.)		Analysis of variance for precooking holding-period
	Control	Papain treated	
0	4.40	3.79	$F = 0.09$[1]
1	4.53	4.04	
5	4.38	3.91	
Student's t-distribution, control vs. papain treated	$t = 5.16$[2]		

[1] Not significant
[2] Significant at 0.1% probability level.

tenderness of the papain-treated samples and that of the untreated controls. These data show that there were no significant differences between treated samples held for various lengths of time before cooking and the samples cooked immediately. Even with the extreme precooking holding period of 5 hours there was no significant effect.

Effect of temperature on papain activity. The activation energy of papain as determined by milk-clotting and by hemoglobin hydrolysis was 16,000 and 8,290 cal. per mole, respectively. Figures 1 and 2 give the Arrhenius plots for the hemoglobin and milk clot methods, respectively.

The products of the hydrolysis of beef by papain. The averages of the nitrogen content of the various fractions of papain-hydrolyzed *biceps*

All samples were put in 10% formalin and evacuated immediately. After 2 days they were dehydrated by a dioxane schedule and ultimately embedded in paraffin. Sections were cut at 10 μ and oriented so that the plane of cut was perpendicular to the surface powdered with papain. Staining was carried out after the method given by Hanson *(4)*.

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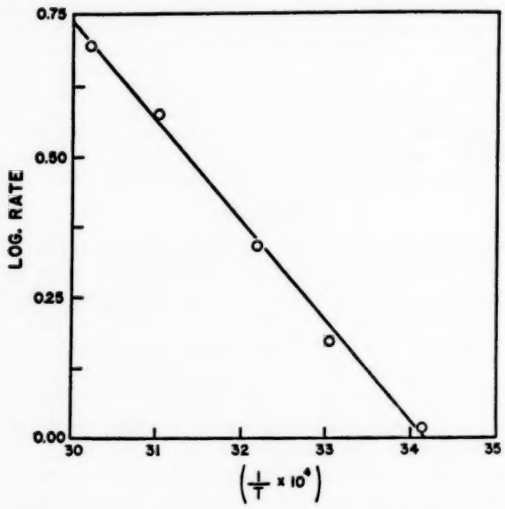

Figure 1. The Arrhenius correlation of the rate of papain catalyzed hemoglobin hydrolysis and the temperature.

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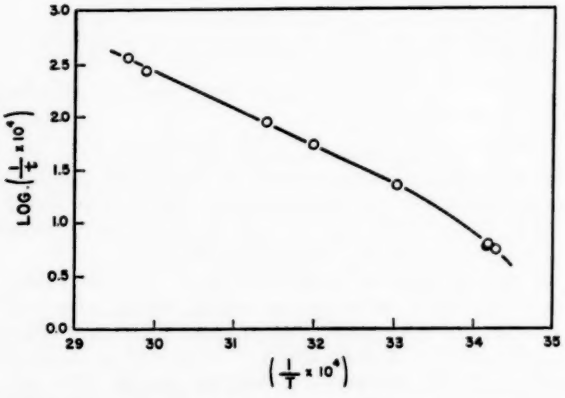


Figure 2. The Arrhenius correlation of the rate of papain catalyzed milk-clotting in reciprocal seconds (t) and the temperature.

TABLE 2

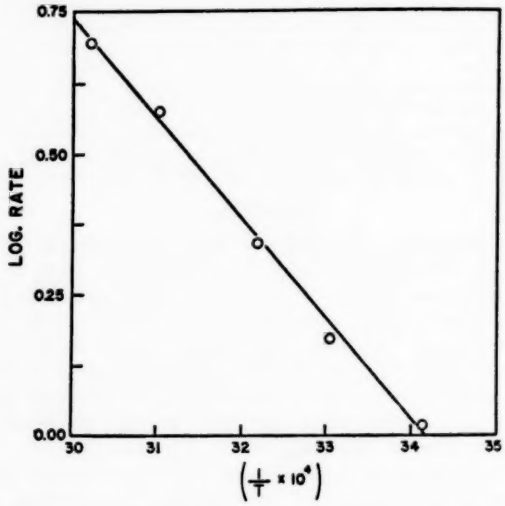
The analysis of beef proteins following papain catalyzed hydrolysis at various temperatures

Protein fraction	Per cent nitrogen, dry weight basis					
	Control 60°C.	0°C.	20°C.	40°C.	60°C.	80°C.
Total soluble nitrogen	11.39	11.38	11.33	11.31	11.85	11.87
Soluble protein nitrogen	9.33	6.07	5.87	5.39	3.54	3.87
Non-protein nitrogen	2.02	5.05	5.45	5.88	7.99	7.71
Elastin nitrogen	0.268	0.093	0.194	0.105	0.088	0.228
Collagen nitrogen	0.521	0.518	0.561	0.477	0.054	0.077
	Weight in grams					
		0°C.	20°C.	40°C.	60°C.	80°C.
Collagen and elastin		0.20	0.29	0.20	0.06	0.12

Effect of heating meat cooked with enzyme powder. When meat coated with enzyme powder was placed in a broiler or in a pressure cooker, definite changes occurred, distinct from those due to heating alone. Figure 3 shows a cross section of the raw meat, with collagenous and elastic fibers among the muscle fibers. Nuclei are readily noticeable at the periphery in the muscle fiber cells. Figures 4-7 represent adjacent muscle segments which were cooked in a pressure cooker. In Figure 4, no enzyme preparation was used. There is a layer of coagulated material over the surface of the meat, and some of the muscle fibers near the surface are beginning to separate from each other. The bounding sarcolemna membranes, which can be seen in Figure 3, are much less conspicuous at the surface of the sample after cooking. Although the nuclei of the muscle fibers are still quite apparent throughout the section, the collagenous fibers have lost much of their stainability. Elastic fibers appear unaffected by the heat treatment.

After application of the enzyme powder and cooking, marked changes are found in the meat tissues. Not only is the sarcolemna destroyed at the surface of the sample, leading to marked separation of muscle fibers, but in some cases these fibers have coalesced (Figures 5-7). Only much deeper in the meat are indications of the sarcolemna to be found. Likewise, the nuclei of the muscle fibers are apparent only at a considerable distance from the treated surface of the meat. Subsequent stages in hydrolysis involve the appearance of small open areas in the muscle fibers (Figure 7). Figure 8 is a longitudinal section, showing stages in the breakdown of the muscle fibers when the papain powder is applied at the exposed ends of these fibers during broiling. Although collagenous fibers at the surface of the enzyme-treated meat have disappeared, the elastic fibers are still intact there and have suffered no diminution in staining intensity.

The coagulum about the fibers in Figures 7 and 8 may represent in part some of the enzyme preparation, but its staining properties suggest that in large part it originates from products of breakdown of the meat tissue.



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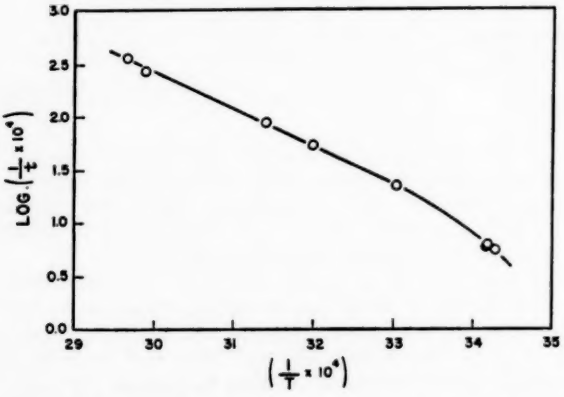

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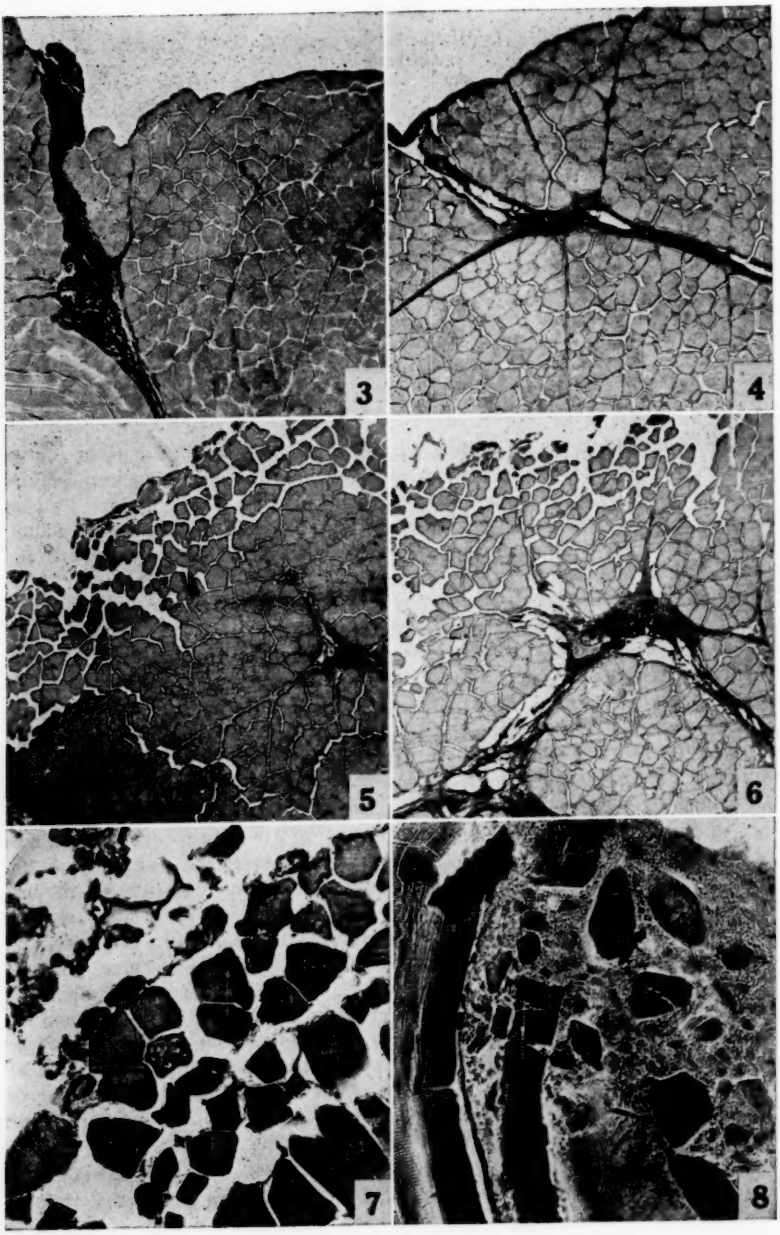
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All sections are of semitendinosus muscle of beef.

Figure 3. Cross section of part of raw muscle. Large dark mass at left is a group of elastic and collagenous fibers. Smaller intercellular strands of connective material are principally collagenous. × 88.

Figure 4. Cross section of part of cooked muscle. Amorphous congealed layer at surface may be denatured soluble proteins. Note that muscle fiber nuclei may be seen up to surface of meat. Collagenous fibers stain less intensely. × 88.

Figure 5. Cross section of part of papain-treated, cooked muscle. Layer of papain is at surface at upper left. Breakdown of intercellular cement is more noticeable than in untreated, cooked muscle. Some cell fusions occurring. Elastic fibers are virtually unchanged. × 88.

Figure 6. Another sample as in Fig. 5. Note loss of stainability in collagenous fibers and their breakdown close to surface. Enzymatic effect in both sections extends to depth of about 0.4-0.5 mm. × 88.

Figure 7. Enlarged photomicrograph of some muscle fiber cells near surface in same piece of meat as in Fig. 5. Debris at upper left is composed of vegetative cell structures, etc., of papain powder. Muscle fibers are beginning to break down immediately below surface. × 312.

Figure 8. Longitudinal section near surface of papain-treated, broiled muscle. Physical destruction of the muscle fibers appears to take place along definite stratification lines in the myofibrils. × 312.

DISCUSSION

The fact that there was no histological evidence of papain penetration or digestion of beef after exposure to concentrated papain for 3 hours at room temperature immediately suggests that mere surface treatment with papain is ineffective and that precooking holding periods are unnecessary.

Further evidence of the limitations of papain penetration at temperatures of from 39° to 55°C. were obtained from the dye tracer studies. In these studies it was assumed that the relatively small dye molecules would readily diffuse to any depth to which the large papain molecules could penetrate. The maximum penetration of dye to about 2 mm. clearly indicates that effective distribution of papain within the meat cannot be accomplished by natural diffusion or by papain's hydrolytic activity. The histological study of beef cooked with papain also shows that papain does not affect sarcolemma, nuclei, or muscle fibers at distances greater than 0.5 mm.

Together, these 3 studies indicate that the incorporation of papain into meat to be tenderized is a most important problem. Physical means of incorporation should be used. The technique of thoroughly forking papain into the meat may be a suitable solution to this problem.

The determinations of activation energies for papain-catalyzed hemoglobin hydrolysis and milk clotting give the quantitative effect of temperature on the catalytic energy of 8,290 cal. per mole for hemoglobin hydrolysis and the activation energy of 16,000 cal. per mole for milk clotting indicates large fundamental differences between these two reactions. Hemoglobin hydrolysis is more similar to meat tenderization. Based on the activation energy for papain-catalyzed hemoglobin hydrolysis, the relative activity of papain at 60°, 70°, and 80°C. compared with 20°C. is 5.5, 8.0, and 11.3, respectively. If only papain's activity in hydrolyzing proteins as a function of temperature is considered meat tenderization should be considerably greater during the cooking period than at room temperature.

However, studies of the effect of temperature on the hydrolysis of beef *biceps femoris* showed that the reaction transforming soluble proteins into amino acids was great at 60° and 80°C. compared with the lower temperature at 20°C. and that this reaction did not follow the Arrhenius equation. Also, hydrolysis of both elastin and collagen was greatest at 60°C. It is apparent that the effect of temperature on the reactants is important in determining the conditions for maximum tenderization. In the temperature region 60° to 80°C., the muscle-fiber proteins, mainly actomyosin, would be denatured and more readily hydrolyzed by papain. At 60°C., the hydrolysis of collagen to gelatin is an important factor in collagen's greater susceptibility to papain-catalyzed hydrolysis.

Although these chemical data on the effect of temperature on hemoglobin and beef hydrolysis indicate the major tenderization should occur at the higher temperatures of cooking, the most critical examination of the need for a precooking holding period was the study of the papain tenderization of samples of beef *semitendinosus*. The data in Table 1 show that papain treatment gave definite tenderization but that the holding periods of 1 and 5 hrs. before cooking had no significant effect. Therefore, the significant tenderization must have occurred during the cooking period. Collectively, the studies on the histology of papain action, the effect of temperature on papain activity and the hydrolysis of beef proteins, and the effect of precooking holding periods on beef tenderization indicate that precooking holding periods are unnecessary.

The studies comprising this paper give some insight into the mechanism of papain tenderization of beef. Probably the most important tenderization mechanism is the hydrolysis of muscle-fiber protein, which accounts for three-fourths of the edible portion of beef. Papain hydrolyzes the sarcolemna and the muscle cell nuclei before there is any apparent digestion of the muscle fibers themselves. As measured by the transformation of soluble protein to amino acids, papain hydrolysis reaches a maximum at temperatures of 60° to 80°C. It is probable that the heat-labile muscle proteins denature before the relatively heat-stable papain and then papain hydrolyzes these denatured proteins with maximum effect.

Since both elastin and collagen contribute to the toughness of meat, it is very significant that papain catalyzes their hydrolysis with maximum effect at 60°C. The different behaviors of elastin and collagen shown in Table 2 suggest either that elastin and collagen may be hydrolyzed by different enzymes in the papain mixture or that their mechanisms of hydrolysis by papain are very different. Probably the latter is true because at temperatures of 60° and 80°C. the hydrolysis of collagen to gelatin would greatly speed up the apparent hydrolysis of collagen by papain.

Thus, tenderization by papain cannot be ascribed to one specific reaction but rather to a general hydrolysis of all of the structural components of beef muscle.

SUMMARY

Histological and dye tracer studies showed that papain penetration into beef is limited to 0.5 to 2 mm.

Papain-catalyzed hemoglobin hydrolysis and milk clotting have activation energies of 8,290 cal. per mole and 16,000 cal. per mole, respectively.

The greatest hydrolysis of beef proteins catalyzed by papain occurred at 60° and 80°C.

Precooking holding periods of 1 and 5 hours had no significant effect on tenderization of beef with papain.

Tenderization with papain is ascribed to the hydrolysis of all structural and functional components of the beef muscle.

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MECHANISM OF COPPER CASSE FORMATION IN WHITE TABLE WINE. I. RELATION OF CHANGES IN REDOX POTENTIAL TO COPPER CASSE

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Food Technology Department, University of California, Berkeley

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The formation of turbidity or haze in table wines and the subsequent precipitation of brownish-red amorphous material has been ascribed to the formation, flocculation, and precipitation of colloidal cupric sulfide *(34)*, cuprous sulfite *(33)*, or proteins *(23, 24)*.

Ribéreau-Gayon *(34)*, in his early investigations of the factors affecting copper casse formation, concluded that the following conditions are required: copper in concentration higher than 0.5 or 0.6 mg./liter; presence of substances capable of flocculating colloidal cupric sulfide; and an oxidation-reduction potential lower than 0.18 or 0.20 volts. Deibner *(8)* subsequently confirmed these results and concluded that while formation of colloidal copper precipitates in wine is related to the redox potential, it is not sufficient to explain hazes of this type, because other chemical and physical factors are involved.

Changes in the oxidation-reduction (redox potential) occurring during the fermentation and storage of wine, measured by both potentiometric and colorimetric methods, have been reported. During 1931-1953, however, these have been limited largely to changes occurring during fermentation, aging, treatment, and storage of wine *(5, 8, 9, 11, 12, 15, 19, 20, 25, 34, 36, 37)*. In general it has been shown that during fermentation the redox potential drops to a low level characteristic of the strain of yeast and method of fermentation, then rises during transfer, racking, filtration or clarification, and later during storage in closed containers decreases again. Wine, whether stored in large containers or bottles, tends to become more reducing.

Wine is very poorly poised in redox potential and exposure to air and absorption of oxygen rapidly increases the potential *(1, 15, 19, 20)*.

The actual nature of the oxidation-reduction systems present and their contribution to the redox potential are still unknown. Various organic constituents (tannins, anthocyanins, ascorbic acid, carbohydrates, and their breakdown products) and metallic constituents (iron and copper) have been implicated *(3, 9, 13, 14, 34, 38)*.

The effect of metal ions, copper and iron, on the redox potential of wine was particularly studied by Ribéreau-Gayon *(34)*. Since the redox potential for the cupric-cuprous couple is about 0.18 volts at pH 7, which is very near the limiting potential of reduced wines, he conjectured that then the reduction of cupric copper in wine is very difficult and is accomplished only long after the disappearance of the dissolved oxygen. The potentials he observed in aerated wine did not permit the presence of more than a trace of ferric ion. This and other evidence led him to conclude that most of the ferric ion was not free but present as a complex.

The interpretation of redox potential changes in terms of basic oxidation-reduction reactions involved is difficult because direct measurement of the characteristic potentials of such systems cannot be made. The commonly accepted values of the redox potential of the cuprous-cupric and ferrous-ferric couples as given by Latimer *(26)* are based on inference from thermodynamic data, rather than actual experimental measurement. Dawson and Nair *(6)* applied the copper amalgam electrode in the investigation of the chemical nature of the copper complexes in peat soils and plants. Subsequently they experienced great difficulty in the use of the copper amalgam electrode *(7)*. This was partly explained by Tourkey and El Wakkad *(39)* who studied the equilibrium between cuprous and cupric ions and behavior of coppor electrodes in dilute copper solutions. They concluded that copper electrodes were unstable in solutions more dilute than about 10^{-4} molar.

Direct measurement of the distribution of iron and copper ions present in wine into reduced and oxidized states, free or combined, also, has not been reported. While the distribution of iron ions into free, ferrous, and ferric forms, and combined ferrous and ferric states can be determined qualitatively and has been determined fairly quantitatively by Ribéreau-Gayon and others *(29, 34)*, the distribution of copper as cupric and cuprous ions has not been possible. Only recently have sensitive colorimetric methods been suggested for the determination of cuprous ions *(31, 40)*, and these have not been applied as yet to the determination of cuprous ion in presence of cupric.

Investigations of the effect of various factors on copper casse formation in white table wines were carried out in our laboratory during 1951-1954. These were reported in detail by Lukton *(27)* and presented in part at a recent meeting of the Technical Advisory Committee of the Wine Institute *(32)*. This paper presents our data on redox potential changes which occur during copper casse formation.

EXPERIMENTAL PROCEDURE AND RESULTS

Potentiometry and turbidimetry. At first, changes in redox-potential in wines susceptible to copper casse formation were measured and changes in turbidity were observed visually. This proved to be inadequate because the time when turbidity first appeared could not be accurately determined and extent of clouding could not be measured. Therefore, a turbidimeter was constructed and the turbidity and changes in redox potential were measured simultaneously as described below.

The wines to be tested were brought to room temperature, filtered if not clear, adjusted to the desired component concentration, saturated with air to insure constant oxygen content and transferred to the electrode vessels. Four-ounce, wide mouth, soft glass jars, 4.7 cm. in diameter and 11.3 cm. high, were used as reaction vessels. They were selected to accommodate a No. 8 rubber stopper which contained 5 holes and which had previously been boiled in concentrated sodium hydroxide and washed thoroughly in distilled water. Two platinum wire electrodes, a saturated potassium chloride–three per cent agar salt bridge and a tube leading to a stoppered vial containing 10 ml. of saturated pyrogallic acid solution in which rested another tube which contained 7 ml. of 20% sodium hydroxide, were inserted. After the bottles were filled with wine at room temperature and stoppered, oxygen-free nitrogen gas was passed through the system to sweep out the air. Then the unoccupied hole in the stopper was stoppered with a glass rod and the pyrogallic acid tube was also quickly stoppered. Thus the head space was practically free of oxygen. To ensure its complete removal, the sodium hydroxide was tipped into and mixed with the pyrogallic acid resulting in a highly

reducing system. The upper one-third of each bottle was painted with black paint on the outside to minimize stray light effects on the turbidity measurements. The salt bridges were made of heavy walled capillary tubing with an inside diameter of 1 mm. In order to prevent slippage of the agar gel and to minimize the deterioration of the salt bridge itself, each end of the tubing was tapered to an inner diameter that was as fine as was practicable in getting the KCl-agar gel into the bridge while hot. Instead of storing the exposed end of the bridge in a saturated KCl solution to prevent drying out, it was found to be more desirable to cover the tip with a rubber policeman and to soak it in the KCl solution in order to make electrical contact before measuring the potential. The platinum electrodes were made of bright platinum wire, 26 gauge, spirally wound and sealed in pyrex glass tubes. The platinum was welded to fine copper wire which emerged from the top of the tubes through a film of de Khotinsky cement seal used to prevent short circuit at this point. The electrodes were cleaned with nitric acid and washed with distilled water prior to their use.

The potential of the system—platinum wire/wine/saturated potassium chloride agar/saturated potassium chloride solution/saturated calomel cell—was measured with a research model Leeds and Northrup No. 7661-A-1 glass electrode potentiometer. The electrode potentials usually agreed within 5 millivolts. Occasionally large differences were observed and the cause could be traced either to a poor platinum-glass seal or a broken copper-platinum weld. The bottles were stored in diffuse light at room temperature which averaged 22°C. ± 2°, and readings were taken at one- or two-day intervals. Unless otherwise stated, the bottles were not agitated to disperse the settled deposit.

The light scattering apparatus for measuring turbidity was constructed on the basis of a circuit suggested by O'Konski *(30)* to accommodate the electrode vessels described above. The instrument was designed to give a quantitative measure of turbidity changes and was not intended as a measure of absolute light scattering intensity in relation to particle size and numbers. All turbidity readings given below were obtained relative to distilled water with which the instrument was set on zero.

Types and composition of wines. The wines used were selected to provide wide variation in composition, particularly in protein and sulfur dioxide content. Their composition is shown in Table 1. Wine 1, a commercial wine high in sulfur dioxide content and low in protein, showed a notable tendency to form a copper casse. Wines 2 through 5 contained larger quantities of protein but were prepared by the Enology Department (this University) with minimal quantities of sulfurous acid.

Wine 1 was varied over a wide range in its chemical composition. Copper, iron, and sulfurous acid were adjusted to various concentrations as shown in Table 2.

At copper, iron, and sulfurous concentrations of 3.0, 4.6, and 333 mg./liter, respectively, the following variations were made: dextrose, 1.5 and 3.0 g./100 ml.; fructose 1.5 and 3.0 g./100 ml.; tannic acid U.S.P. 0.2 and 0.5 g./100 ml.; phosphate (Na_2HPO_4) 0.2 and 0.5 g./100 ml.; ethyl alcohol, to 15 and 20%; ethyl alcohol 15 and 20% and 3 g./100 ml. of invert sugar; pH, 3.35, 3.30, 2.54, 3.98, 4.96; citric acid, 0.5 g./100 ml. to pH 3.00; malic acid, 0.5 g./100 ml. to pH 3.02; tartaric acid 0.5 g./100 ml. to pH 2.90; phosphate (Na_2HPO_4) 0.5 g./100 ml. to pH 4.17; malonic acid, 0.2 g./100 ml.

TABLE 2

Variation in composition of Wine 1.

Sample	Cu mg./liter	Fe mg./liter	SO_2 mg./liter
1	0.5	4.6	333
2	3.0	10.0	333
3	5.0	4.6	333
4	5.0	10.0	333
5	0.5	4.6	400
6	0.5	4.6	500
7	3.0	4.6	400
8	3.0	4.6	500
9	5.0	4.6	400
10	5.0	4.6	500
11	3.0	4.6	333

TABLE 1

Composition of wines used

Wine	Type	Total acid as tartaric g./100 ml.	Volatile acid as acetic g./100 ml.	Ethyl alcohol vol. per cent	Extract g./100 ml.	Reducing sugars g./100 ml.	Phosphate mg./PO_4/100 ml.	Tannin mg./100 ml.	Sulfur dioxide mg./liter		pH	Copper mg./liter	Iron mg./liter	Protein 10% TCA ppt. mg./liter
									Free	Total				
1	Sauterne	0.600	0.048	12.4	....	0.810	49.3	34.0	148	333	3.30	0.5	4.6	15.4
2	French Colombard	1.05	0.054	10.5	3.1	0.10	33.6	19.0	1.5	16.5	3.40	0.10	2.0	30.6
3	Semillon Blanc	0.86	0.036	13.1	2.7	0.09	9.12	15.0	1.1	9.0	3.12	0.23	5.2	98.0
4	Clairette Blanche	0.77	0.043	11.3	2.6	.....	31.7	15.75	1.0	9.2	3.40	0.0	2.1	111.0
5	French Colombard	0.83	0.033	14.0	2.5	0.07	21.9	19.0	1.1	7.5	3.25	0.0	5.3	.....

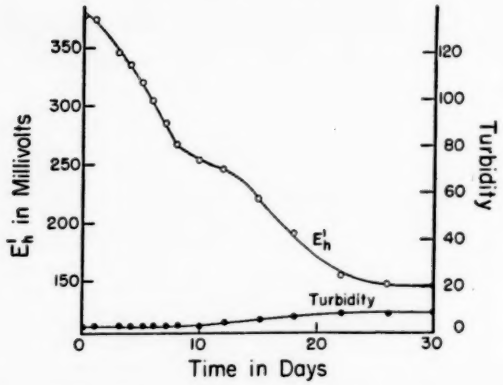

Figure 1. Changes in redox potential and turbidity at room temperature at 0.5 mg. per l. of copper.

and 0.5 g./100 ml. to pH 2.99 and 2.73, respectively; Versene 1 and 2 equivalents per metal equivalent; starch, pectin, casein, gelatin at 10 mg./100 ml. each. The metal ions were removed by blue fining and added back as follows: Copper 0, 3.0, and 5.0 mg./liter at an iron content of 0.4 mg./liter; copper 3.0 and iron 10.0 mg./liter.

Analytical methods. The alcohol, sugar, acid, extract, tannin, phosphate, sulfur dioxide, total copper, and iron content were determined by procedures described previously *(21, 22)*. Sulfate was determined by the usual gravimetric barium sulfate precipitation procedure after expelling sulfur dioxide from the acidified wine by heating in presence of carbon dioxide. Protein was determined as the 10% trichloracetic acid precipitable nitrogenous matter. The wine after additions of trichloracetic acid was heated at 90°C. for 15 minutes, centrifuged, and sediment washed twice with 10% trichloracetic acid *(18)*. The nitrogen content of the precipitate was determined by the microkjeldahl method *(2)*.

Effect of metal and sulfur dioxide level. The addition of iron in the range of 4.6 to 10.0 mg./liter and sulfur dioxide in the range of 333 to 500

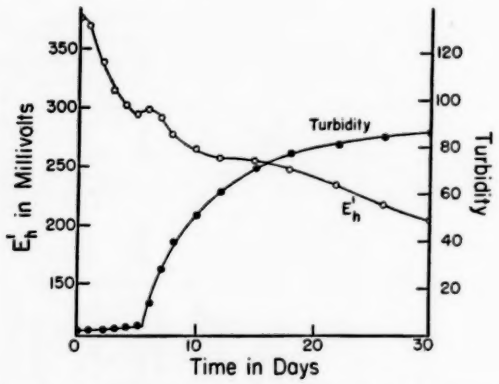

Figure 2. Changes in redox potential and turbidity at room temperature at 3.0 mg. per l. of copper.

mg./liter had no effect on the redox potential or turbidity changes. Measurable turbidity did not occur in samples containing 0.5 mg./liter copper, as shown in Figure 1. Increasing the copper content to 3.0 mg./liter, however, resulted in marked increase in turbidity as shown in Figure 2. Figure 2 is typical of the results obtained with wines containing copper at a level of 3 mg./liter or higher, at a pH of 3.3. The redox potential drops rapidly at first without appreciable change in turbidity, then just before turbidity begins to increase the redox potential rises and then drops again. This rise in redox potential just before formation of the particles responsible for turbidity always occurred with wine 1, but did not occur or was not as pronounced with other wines. With this exception the changes in redox potential during casse formation obtained with all wines tested were similar. The redox potential at the time of the initial appearance of copper casse was 295 ± 10 millivolts in all wines tested at pH 3.30.

Effect of EDTA. The addition of ethylene diamine tetra acetate (Versene) as a chelating agent caused a rapid drop in potential and inhibited turbidity formation. The redox changes in the presence of one equivalent of versene per metal equivalent are shown in Figure 3. The binding of

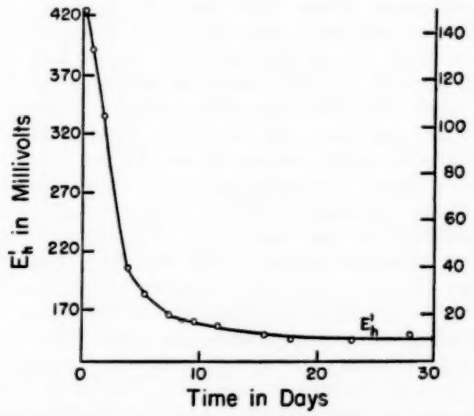

Figure 3. Changes in redox potential in presence of Versene.

cupric or ferric ions which resulted in suppressing their contribution to the over-all redox potential of wine is clearly demonstrated. The effect of EDTA on copper and iron casse has been reported previously *(21)*. Ching *(4)* recently reported that EDTA prevented precipitation of cupric copper and of ferrous iron, but not ferric ion by potassium ferrocyanide.

Effect of pH. The pH in the range of 2.5 to 5.0 had a marked affect on the redox potential. The changes in redox potential with time at various pH values, shown in Table 3, indicate that the redox potential at which turbidity was first observed decreased by a fairly constant amount with increase in pH, on the average of 69 mv. per pH unit.

Effect of other factors. The presence of the added colloids, starch, pectin, casein, and gelatin had no effect on the potential at which turbidity

TABLE 3

Effect of pH on redox potential changes.

pH	E_h' (millivolts)				
	Days stored				
	0.25	3.8	7.5	11.6	15.6
2.5	445	367	323[1]	315	315
2.9 (tartaric acid)	427	317	309[1]	310	306
3.0 (citric acid)	414	319	302[1]	294	284
3.3	400	329	286[1]	287	284
4.0	303	257	249[2]	235	223
5.0	259	163	155[2]	135	116

[1] Potential at which turbidity first appeared.
[2] There was no turbidity formation at these pH values.

formed. However, the removal of colloids (protein) originally present by blue fining inhibited turbidity formation but had no effect on the potential changes (not all of the heavy metal ions was removed by this treatment). Addition of dextrose, fructose, ethyl alcohol, phosphate, citrate, malate, tartrate, and malonate had no notable effect on potential changes.

Effect of sulfur dioxide. To investigate the effect of sulfur dioxide at very low concentrations, wines were obtained which were produced with a minimum of added sulfurous acid and which had a final sulfur dioxide concentration of from 7.5 to 16.5 mg/liter (wines 2, 3, 4, 5—Table 1).

These wines were set up and treated as before except that 10 mg./100 ml. of benzoic acid was added as preservative. Copper was varied from essentially zero to 5 mg./liter and the sulfur dioxide was adjusted to 100 and 300 mg./liter at a copper concentration of 5 mg./liter.

At 5 mg./liter copper, the redox potential values at the appearance of turbidity are shown in Table 4. These results indicate a redox potential range of 55 millivolts, or an average of 290 ± 30 millivolts. There was little or no effect when the sulfurous acid content was increased from 100 to 300 mg./liter. The pH of the wine had a more marked effect on the redox potential at which casse began to form than did the sulfur dioxide content.

Effect of sunlight on redox potential in wine. Two series of reaction vessels were prepared; one painted completely black except for a fine strip to allow light to pass through in measuring turbidity (this was covered with tape except when measurements were being made), and another set was not painted. Wine 1 at a copper concentration of 5 mg./liter was used. The electrode vessels were prepared as before and exposed to direct sunlight during the day (light period). During this time there was a noticeable increase in temperature but the difference between the two sets was

TABLE 4

Effect of sulfur dioxide on the potential at which turbidity forms.

Wine	pH	E_h'	
		SO_2 (mg./liter)	
		100	300
2	3.40	277 mv.	260 mv.
3	3.10	315	315
4	3.40	272	272
5	3.25	305	305

small, at most 3°C. higher in the dark bottles. At the end of each day the bottles were returned to the laboratory and during the evening (dark period) the temperature of the wines dropped to that of the room. Redox potential and turbidity measurements were taken at the beginning and end of each period.

Changes in turbidity and redox potential under these conditions are shown in Figure 4. These data indicate the reducing effect of light energy

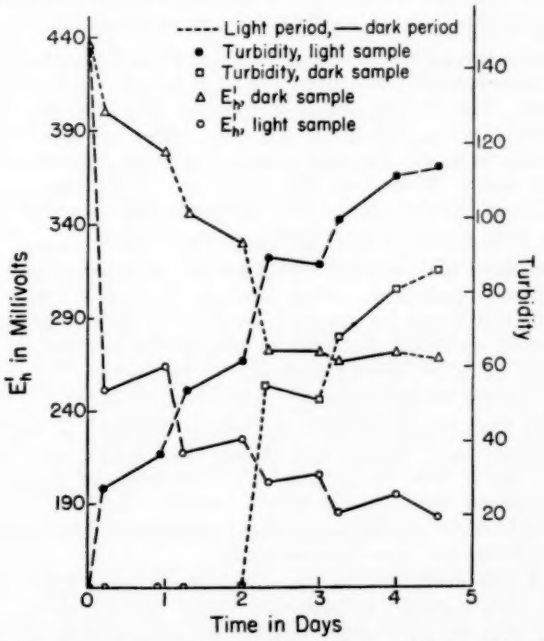

Figure 4. Effect of exposure to light on redox potential and turbidity.

and suggest that a photochemical reduction as well as a non-photochemical reduction mechanism is involved in the formation of copper casse.

In a wine the hydrogen atoms which may be produced by the photochemical reduction of sulfite would serve as reductant in the production of cuprous ions or in the formation of sulfide ions from sulfurous acid, thus causing a rapid drop in redox potential.

At the pH of wine, very little sulfite ion exists, but instead, bisulfite ion is the main ionic specie. Soft glass transmits ultraviolet light energy only at the upper end of the ultraviolet range, about 3200 to 4000 Å, where the energy of radiation is considerably less than at 2655 Å. However, these reactions may occur to some extent in wine and present interesting possibilities for further investigation.

Synthetic solutions. A stock solution was prepared containing 15% ethyl alcohol (by volume), 7.5 g./liter tartaric acid adjusted to pH 3.5, 250 mg./liter sulfur dioxide and 5 mg./liter cupric ion.

The electrode vessels were assembled as in the case of the wines. Various substances were added: sucrose, dextrose, and fructose at 1, 5, and 10 g./100 ml. levels of each; iron, 5 and 10 mg./liter; iron, 5 mg./liter and glucose, 1 g./100 ml.; iron, 5 mg./liter and glucose, 10 g./100 ml.; iron, 10 mg./liter and glucose, 1 g./100 ml.; iron, 10 mg./liter and glucose 10 g./100 ml.; tannic acid U.S.P., 0.01, 0.05, and 0.10 g./100 ml.; grape seed tannin, 0.01, 0.05, and 0.10 g./100 ml.; combinations of iron, glucose, tannic acid, albumin with and without added sodium metabisulfite, sodium sulfite, sodium hydrosulfite, glucose bisulfite, acetaldehyde bisulfite.

The samples were stored at room temperature in diffuse light and redox potential measurements taken over a 20-day period. In no case did turbidity appear. Only in the sample containing 10 g./100 ml. fructose and that containing 0.10 g./100 ml. grape seed tannin the potential dropped to a low enough potential for copper casse formation, according to the data observed for wines. These were $E'_h = .302$ m.v. at 20 days and 252 m.v. at 12 days, respectively. In general the potentials were far above those observed in wine during a comparable period of time.

Using the same basic solution, Ribéreau-Gayon reported a copper clouding when it was exposed to intense sunlight for a few weeks. The same phenomenon, which will be reported elsewhere *(28)* was observed by us. The most likely reducible materials in this system are copper and sulfur dioxide, both of which are present in the precipitate. Thus the photochemical reduction of sulfurous acid must be considered.

That this reduction is possible has been established as a result of the early investigations of Franck and Haber *(10)*, Haber and Wansbrough-Jones *(17)*, which were re-evaluated by Rollefson and Burton *(35)*. In this photochemical reduction, hydrogen, sulfate ions, and dithionate ion are produced and it is possible that cupric sulfide may form from the latter but so far this has not been demonstrated.

Effect of temperature. The electrode vessels were filled with wine 1 and stored in a constant temperature water bath at 45°C. They were adjusted so that the salt bridge was not in contact with the water bath. The wine was varied in copper from 0.5 to 15 mg./liter and a control series stored at room temperature was set up in the same way. The bottles were agitated before each reading to disperse the deposits. After storage for 20 days, the samples at 45°C. were saturated with air and changes of redox potential and turbidity were observed.

Data for copper at a concentration of 3.0 mg./liter are indicated in Figure 5. The potential time curves were similar at all copper concentrations at 45°C. and data at room temperature corresponded to those previously obtained for the same wine, therefore the data are not listed here, except for those given in Table 5.

Assuming the reaction to be essentially complete at the end of 10 days at 45°C. and at 50 days at 22°C., the half time of the reaction estimated from the data plotted is shown in Table 5.

The large difference in rate of decrease in redox potential at 45°C. as compared with that at 22°C., indicated a large temperature coefficient. This was also true for the rate of turbidity formation. The intensity of

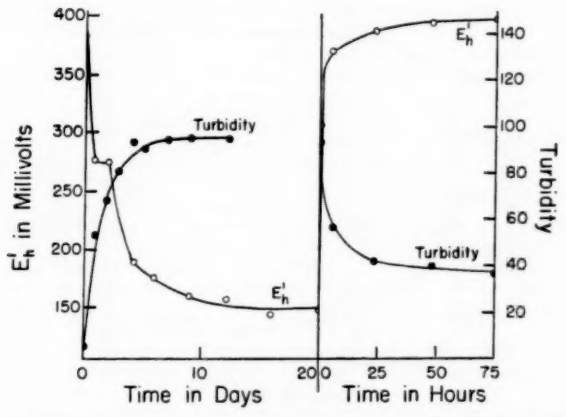

Figure 5. Changes in redox potential and turbidity at 45°C. under anaerobic (left) and aerobic (right) conditions. Illustrating changes occurring during formation and solution of copper casse.

the maximum turbidity observed was found to be copper dependent; on the other hand, the rate of turbidity formation at 45°C. was not.

The data on turbidity disappearance (Figure 5, all samples at 45°C. gave similar curves) indicated, as did those at 22°C. (see Figure 6), the lack of dependence of rate of potential and turbidity change on copper concentration. The rate of disappearance, on the other hand, is notably affected by temperature.

SUMMARY AND CONCLUSIONS

Variation of copper, iron, sulfur dioxide, tannin, fructose, dextrose, sucrose, ethyl alcohol, phosphate, malate, citrate, and malonate over a wide range of concentration resulted in little or no change in the redox potential at which copper casse forms. There was variation, however, among wines over a 55 millivolt range in the potential at which casse began to form. This could be explained partly as a pH effect.

TABLE 5

Effect of copper content and storage temperature on rate of casse formation.

Copper mg./liter	Final turbidity intensity	Half-time in days
	at 45°C.	
0.5	54	0.9
3.0	94	0.8
5.0	111	0.8
15.0	165	1.0
	at 22°C.	
0.5	....	....
3.0	60	20
5.0	63	15
15.0	71	12

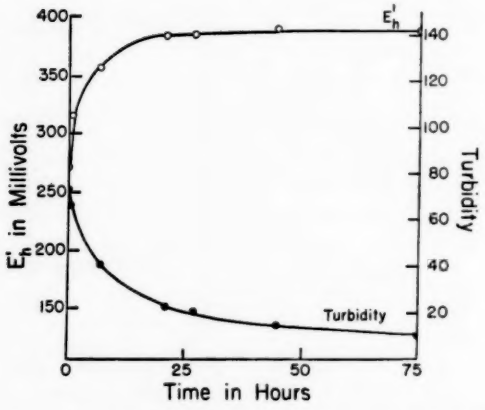

Figure 6. Changes in redox potential and turbidity during solution of copper casse at room temperature.

The influence of pH was marked and the change in potential per pH unit in one wine was about 69 millivolts, in both the oxidized and reduced states of the wine. In the oxidized state the important pH dependent couple is the oxygen-water couple; however, in the reduced state, the pH dependent reducing couple, R-RH is unknown.

Studies on synthetic wine solutions eliminate the possibility that the reducing couple is either sulfur dioxide or the tannins.

By chelating metal ions, whether oxidized or reduced with Versene, the large contribution of the metal ion redox couples to the total redox potential was demonstrated, either as couples or as catalysts of redox equilibrium reactions in the wine. This was manifest in the comparison of the rapid approach and attainment of the limiting redox potential in the presence of Versene in contrast to the slow and gradual change in potential in its absence. The large effect of small amounts of copper and iron salts on the potential showed how poorly poised wine is as a redox system.

The effect of temperature on the rate of redox potential change indicated a large temperature coefficient.

The role of sunlight energy in the copper casse reaction appeared to be of a reductive nature. The light energy may either activate or catalyze the reactions that would occur in its absence, or it may produce reducing species by a photochemical reaction mechanism. Since it is possible to produce copper casse in a synthetic solution in the presence of sunlight and not in its absence, the latter inference seems likely.

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CONTINUOUS RECORDING OF GEL DEFORMATION IN CREEP AND RECOVERY

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When gels of high polymeric materials *(e.g.*, starch, pectin) are placed under stress, there occurs an initially rapid deformation. However, unlike distortion in materials of nearly ideal (Hookean) elasticity, the deformation continually increases (although the rate of deformation gradually falls off) with the time under stress ("creep"). When the stress is released, the reverse process occurs: an initially rapid recovery, which is followed by a gradually decreasing rate of recovery.

In order to record these changes, during creep and recovery, for rheological analysis, it is convenient to use an apparatus which shows the continuous deformation with time. Since some frictional resistance is involved in an apparatus such as that of Davis *(1)* or Press *(3)*, this friction should be eliminated for a strict accounting of the behavior of the material. On the other hand, use of ocular methods of measurement *(2)* results in the loss of continuity of the deformation changes, particularly during the initial stages.

APPARATUS

The principle of the apparatus developed here is that of recording the progressive masking (or uncovering, respectively) of a beam of light by the agency of the deforming body. The light beam falls on a photoelectric cell or phototube, which is a resistance arm in a Wheatstone bridge. The output from the bridge circuit is amplified and applied to a millivolt recorder.

Although the agency of masking may be developed in various ways, the one used here (Fig. 1) is an opaque screen *(SCR)* on one pan of a balance *(B)*. The cylindrical gel sample *(S)* is placed upright on the other pan of the balance, and the balance is adjusted to the zero position with counter weights *(CB)*. A vertically movable piston *(P)*, with a flat surface, is lowered to contact the upper surface of the gel cylinder. This causes a slight deflection of the balance from the zero position and puts a small stress (about 80 dynes/cm.^{2}) on the sample. Then the piston is fixed in position by a clamp spring *(CL)*.

A loading weight *(L)* is placed on the pan which holds the screen. As the gel is deformed, the screen moves downward, allowing more light to pass over the top of it through the rectangular aperture *(A)*. The reverse movement takes places when the load is removed.

OPTICAL SYSTEM

The light source *(LS)* is a 100 watt (125 volt) projector bulb in a Bausch and Lomb microscope lamp housing (Fig. 2). A concave mirror

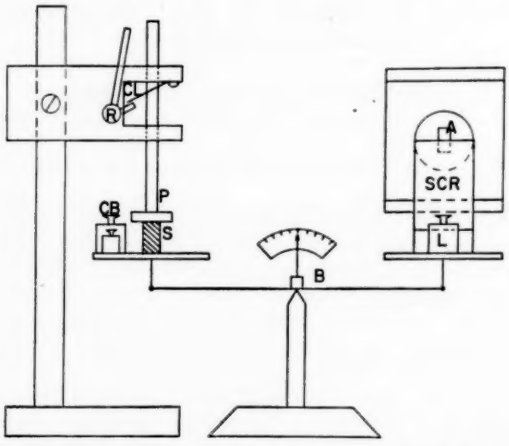

Figure 1. End view of apparatus. CL is the clamp spring, which can be unclamped by lever release R, permitting piston P to be adjusted to any height. CB indicates the weights used to counter-balance the screen, SCR, on the opposite pan of the balance, B. S is the gel sample, which is placed under compressive load by putting the weight, L, on the opposite pan. Movement of the screen regulates the size of the opening, A, through which light passes to the phototube.

is mounted behind the lamp bulb, and the light rays pass through two plano-convex lenses, a ground glass filter, and a clear blue filter. The condensing system *(E)*, which focuses the light beam on the plate of the phototube *(PH)*, is an enlarging lens (focal length = 161 mm.) 180 cm. from the light source. The distance from enlarging lens to phototube is 20 cm.

The screen *(SCR)* is placed 1/8 inch in front of the front element of the enlarging lens system. This latter has its opening restricted to a central rectangular aperture *(A)*, 1 cm. wide by 2 cm. high, by the use of an

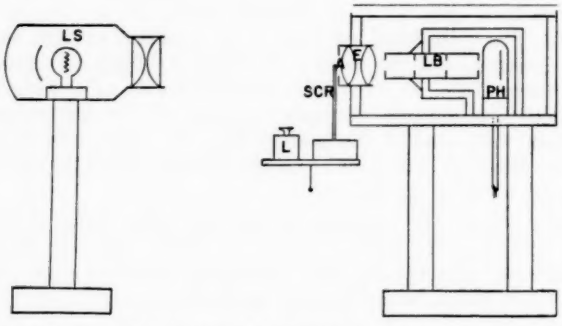

Figure 2. Side view of apparatus. LS represents the light source. L, SCR, and A are as noted in the explanation of Figure 1. E is the enlarging lens system, which focuses the light beam on the sensitive element of the phototube, PH. A light baffle, LB, is interposed in the system to screen out extraneous side light. The wire from the phototube passes directly to the amplifier.

opaque cap. As the screen moves past this rectangular aperture, the intensity of the image on the plate of the phototube changes. A linear relationship is maintained between this intensity change and the vertical movement of the screen, according to the following relationships:

$$I \propto \frac{1}{f^2}, \qquad (1)$$

where I is the intensity of the light beam,
and f is focal length/diameter of lens opening.
Hence, $I \propto (\text{diameter})^2$, (2)
or $I \propto$ area of the lens opening.

Since the aperture is rectangular and since its base remains constant in size, the area of the aperture varies directly with the length of its vertical side.

$$\therefore \quad I \propto \text{vertical position of the screen.} \qquad (3)$$

The light projection system is protected against variations in line voltage by the interposition of a constant voltage transformer (115 volt output) between the line and the auto-transformer which supplies current to the projector bulb. The auto-transformer, as will be explained below, is adjusted to supply 65 volts to the projector bulb.

ELECTRICAL SYSTEM

The phototube circuit and the amplifier are shown in Figure 3. In the

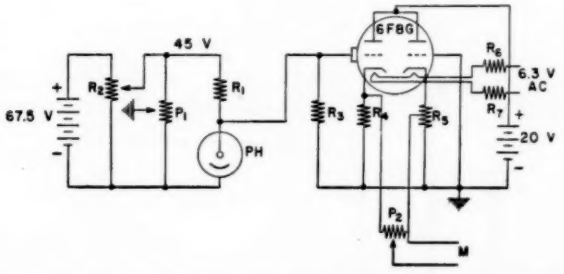

Figure 3. Phototube circuit (left) and amplifier (right). In the phototube circuit, a Wheatstone bridge is formed of the phototube (PH) as one arm, a resistor of 10 megohms (R_1) as a second arm, and a 5 megohm potentiometer (P_1) comprising the other pair of arms. Current supplied by a 67.5 volt dry cell is reduced to 45 volts by a 40 kilohm potentiometer (R_2). In the amplifier, R_3 is a 60 megohm resistor, R_4 and R_5 are 47 kilohm resistors, and R_6 and R_7 are 1 ohm resistors. These latter are connected to a filament transformer, which supplies a 6.3 volt, alternating current (6.3 V A C). The potentiometer, P_2, has a maximum resistance of 2 kilohms. The 6F8G tube is supplied with a 20 volt direct current from a dry cell. M is the millivolt recorder.

former, a Wheatstone bridge is formed by the phototube *(PH)*, its load resistance *(R_1)*, and a potentiometer *(P_1)*, whose movable arm is grounded. When the bridge is in balance, the phototube anode is at ground potential. By the adjustment of the ground point of the potentiometer arm, the bridge may be balanced at any light intensity.

Since the voltage applied to the bridge is constant (at 45 volts), the phototube operates at a uniform voltage. The output of the bridge is then rectilinear with changes in light intensity. However, the output per unit increment of intensity change will vary directly with the initial light intensity. Because of the danger of having non-linear output responses near the point of saturation of the system, the light intensity is kept at an intermediate level by adjusting the auto-transformer of the light source to supply only 65 volts (see above). In order to avoid "noise" and thermal drift, the load resistances in the bridge are relatively low.

When the applied voltage differs from the bucking voltage obtained from the slide wire within the recorder, the millivolt recorder used here (Brown, 0-1 mv.) has a low input impedance. If the phototube bridge were to supply input to the recorder directly, the former would be so loaded that the response of the recorder would be slow and the zero point would drift seriously. An amplifier is included in the system to avoid these difficulties.

The basis of the amplifier system is a cathode follower circuit *(4)*. This circuit was chosen for its potentially high input impedance (10 to 1000 megohms) and low output impedance (below 5000 ohms). Thus, both electrical circuits are satisfied: the bridge "sees" an impedance of 60 megohms, while the recorder "sees" an impedance below 2000 ohms. The parameters of the amplifier have been chosen to avoid noise, thermal drift, and zero drift. The cathode resistors of the *6F8G* tube are 47,000 ohms,

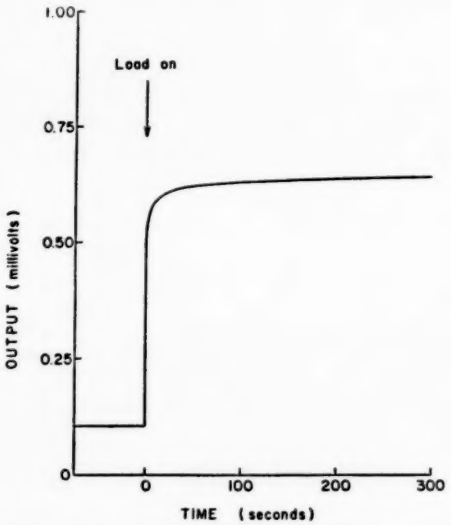

Figure 4. Typical record of deformation of starch gel under compressive stress. Before gel is loaded, initial output of system is 0.105 millivolt here. At zero seconds, load of 50 g. is placed on balance pan. As gel is compressed and aperture is enlarged by downward movement of light screen, output to recorder is increased. The recorder pen then scribes the creep curve depicted.

the plate voltage 20 volts, and the heater voltage 5 volts. The grids are approximately 1 volt negative, with respect to the cathode.

The output of the cathode follower is taken between one terminal and the movable arm of a 2000 ohm potentiometer (P_2), which is connected across the cathode resistors. The potentiometer is connected to these resistors at such points that the voltage output is zero when both grids are grounded. The movable arm of the potentiometer can be adjusted to control the sensitivity of the recorder. At maximum sensitivity, there is approximately one millivolt output per 1 mm. movement of the light screen. In normal operation, the sensitivity is calibrated to 1 millivolt per 10 mm. movement of the screen.

Theoretically, and by actual measurement, the movement of the recorder pen is linearly related to the movement of the screen in front of the lens aperture, which in turn represents the deformation of the gel sample. The zero drift is less than 2% per hour after a 15-minute warm-up period. Figure 4 depicts a sample recording of the behavior of a starch gel under load.

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NOTES AND LETTERS

RECENT MICROBIOLOGICAL ADVANCES IN ENOLOGY

Studies in the field of the microbiology of wine are at present in a state of activity after many years of inactivity. Areas of research concern, as shown by recent studies, are as follows:

Pure and mixed fermentations.
Practical implications of yeast growth.
New developments in antibiotics.

In the observations that follow some suggestions worthy of further exploration are offered.

PURE AND MIXED FERMENTATIONS

Traditionally, a sound fermentation of grape-must is ascribed to the activity of *Saccharomyces cerevisiae*, a variety *ellipsoideus*. Other species of yeast, particularly those of the apiculate group, have always been considered undesirable. For this reason, the practice of adding antiseptic agents to prevent growth of the so-called wild yeast, while permitting the development of selected strains such as *Saccharomyces cerevisiae* variety *ellipsoideus*, has been quite common. This type of race selection is based on different physiological characteristics of the organisms. This poses the question of whether or not those yeasts, which are so different morphologically from the *ellipsoideus* types, are all really undesirable.

Recent observations on these yeasts form a basis for reopening the question. Among the early contributions in this area were those published in Italy by de'Rossi, Verona, and Luchetti. Later the problem was investigated by Castelli and associates and finally by Florenzano, Malan, the writer and others. In France, pertinent contributions were made by Peynaud and Domercq and in America by Mrak and Phaff.

Grapes and grape-must harbor a great variety of yeasts, many of which there is reason to believe are desirable although their metabolic characteristics are not well known. For instance, *Saccharomyces rosei (Torulaspora)* which, in some cases, Castelli and his group properly suggest for use in vinous fermentation instead of *S. cerevisiae* var. *ellipsoideus*, occurs commonly on grapes. *S. rosei* is particularly suitable in cases where a low volatile acidity is desired, but it is not necessary to obtain a high alcohol content. Verona has pointed out that although good results are obtained with pure fermentation, a mixed fermentation might be advantageous and furnish even better results. The commercial index of wine quality is usually based on content of alcohol and volatile acidity.

But this is not really an accurate indication of good wine quality, for other and more complex characteristics are involved. It is possible that a single species or races of yeast will not fulfill all the requirements for good wine making, whereas the use of a mixed fermentation might very well do so. The author, therefore, has suggested two types of mixed fermentations:

Simultaneous mixed fermentations.
Mixed fermentations brought about by gradual mixing of different strains of yeast.

In the first case, it is necessary to study a combination of different species or races of yeasts which are to be inoculated into the must simultaneously. The activity of these two strains must be integrated in order to give the wine the desirable characteristics insofar as flavor, color, smell, taste, and stability are concerned. Unfortunately, studies on such microbial synergism, as related to wine making, have not been developed to the theoretical or applied stage, permitting its extensive use. The few contributions that have appeared on the subject have not presented a solution to the problem. For example, by mixing different strains in different media, Verona found that the associations of two strains may very well lead to a lower alcohol yield. In some cases, there may be a slight reduction in alcohol production and in others there may be a slight increase.

The concept of gradual association was first tried by Verona. The object was to obtain a wine with high alcoholic content but low in volatile acids. In this connection, he observed:

> Yeasts stabilized in a haploid stage (zygosaccharomycetes) produce scarcely any volatile acid.
> Volatile acids are found in largest amounts during the early stages of fermentation.

In view of these circumstances, he suggests the possibility of starting a fermentation with a haploid yeast and, when it reaches a very active stage, adding a diploid strain of *S. cerevisiae* to obtain the mixed fermentation. Verona made his first experiments with *S. veronae* but since the principle has been established, it appears that any other haploid yeast such as *S. rosei* of Cantarelli and Zardetto-Toledo could be used. As a matter of fact, results obtained in such mixtures have been satisfactory in large-scale trials in wine cellars.

PRACTICAL IMPLICATIONS OF THE GROWTH REQUIREMENT OF YEASTS

Studies of the growth requirements of yeasts used in wine fermentations have been made by Wiken and Richard in Switzerland and by Verona and his colleagues in Italy. Although the experiments have not been very extensive, it has been established that:

1. Among the cerevisiae type of yeasts, there are autotrophic and heterotrophic strains. Autotrophic strains are rare.
2. The apiculate yeasts are heterotrophic since they require pyridoxine, mesoinositol, pantothenic acid, and certain other vitamins.
3. Among the other yeasts found in grape-must, some are heterotrophic whereas others are autotrophic.

It should be pointed out that these characteristics in yeasts vary to the extent that they may be partial or conditioned.

The significance of these observations is clear, since they suggest the importance of selecting certain strains of yeast, and the importance of growth requirements as pertains to these particular yeasts. Furthermore, it places due importance on the vitamin characteristics of the must.

In this connection, Ribéreau-Gayon has initiated some very interesting studies concerning the occurrence of thiamine in grape-must. Practical

applications from these studies as well as some on antimetabolites may be foreseen. Verona has suggested the possibility of establishing methods for the formulation of selective conditions in must whereby the growth of such organisms as apiculate yeasts may be prevented; whereas that of cerevisiae strains may be encouraged. For example, if antimetabolites such as anti-pyridoxine, anti-inositol or antipantothenate are added to the natural must, organisms such as *Kloeckera apiculata* and certain species of *Pichia* and *Candida* may be inhibited due to lack of vitamins.

The procedure outlined above has the advantage that it avoids the use of antiseptic agents which are detrimental from the health and organoleptic point of view. This would enable carrying out the fermentation with races of yeasts which can grow under the ecological conditions of the treated medium. Verona therefore advises the consideration of the possible application of antivitamins in the field of enology.

NEW CONTRIBUTIONS IN THE FIELD OF ANTIFERMENTATIVE AGENTS

It is correct to state that sulfurous acid (SO_2) has been an important chemical in enological procedures. But its abuse, more than its use, with respect to hygienic and organoleptic responses has magnified the problem to the point where it is necessary to consider the possibility of replacing that compound with substances less detrimental to human health and the organoleptic characters of wine. The question may appear difficult but it is not impossible to solve. Verona and Ribereau-Gayon and Peynaud have tried using Vitamin K_5 for this purpose. These trials were inspired by the work of Pratt and associates who have already verified the antibiotic action of these compounds on yeast. Verona, Ribéreau-Gayon, and Peynaud have shown that Vitamin K_5 will depress or inhibit wine fermentation. For this reason, Verona has suggested the addition with Vitamin K_5 of small amounts of ascorbic acid to establish reduced conditions.

Other contributions involving the use of antibiotics are concerned with actidione. Verona and Picci have applied the results of Whiffen to wine yeasts. Ribéreau-Gayon and Peynaud have also tried this. Actidione appears to be a very potent anti-fermentation agent. Nevertheless, it is rather selective since it shows a high inhibiting effect on the ellipsoid type of yeast, a slight effect on apiculate ones and no effect on the bacteria occurring in wine. It follows, therefore, that it can be used conservatively with success as an agent for stabilizing wine, particularly sweet wine when fermentation is done. On the other hand, insofar as the bacterial flora responsible for the malo-lactic fermentation are concerned, the compound has no antibiotic properties. Likewise, the acetic acid bacteria can grow.

These investigations are in their infancy but considering the importance of the field is of such general interest, it is hoped that they can be extended to all the countries in which wine production is important.

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